Symposia Biologica Hungarica 14

ULTRASTRUCTURAL FEATURES OF CELLS AND TISSUES IN CULTURE

Akadémiai Kiadó, Budapest

ULTRASTRUCTURE OF CELLS AND TISSUES IN CULTURE
14

ULTRASTRUCTURAL FEATURES OF CELLS AND TISSUES IN CULTURE

edited by

I. TÖRŐ and GY. RAPPAY

(Symposia Biologica Hungarica 14)

Tissue culturing has become one of the basic methods of present day experimental biology. It is the purpose of the annual Conferences of the European Tissue Culture Society to promote the application of this method in the study of all kinds of cell-biological problems.

The papers in this volume are those of the first day of the 1971 meeting held in Budapest and all the contributions are dealing with the fine structure of cultured tissues and cells. The papers cover a wide range of experimental work with objects like lymphoreticular cells, tumour cells, endocrine as well as plant cells. The results indicate that irrespective of whether the original ultrastructure is retained or lost in culture, various functions characterizing the cells *in vivo* are still apparent under *in vitro* conditions. This symposium has been a further proof of the importance of the tissue culture method in studying the control of various vital functions on the cellular level. The electron micrographs presented are especially valuable to cytologists.

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ULTRASTRUCTURAL FEATURES OF CELLS AND TISSUES IN CULTURE

Edited by

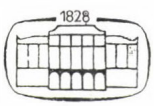
I. TÖRŐ

Department of Histology and Embryology,
Semmelweis Medical University, Budapest

and

GY. RAPPAY

Institute of Experimental Medicine,
Hungarian Academy of Sciences, Budapest



AKADÉMIAI KIADÓ, BUDAPEST 1972

The Symposium was held on the 1st day of the
Meeting of the European Tissue Culture Society
in Budapest, 4th–6th May 1971

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FOREWORD

This volume contains the material of the Symposium held at the 1971 Meeting of the European Tissue Culture Society in Budapest. Hungarian histologists were greatly honoured by being able to welcome this society of great reputation in Hungary. The Symposium offered a valuable occasion for presenting reports on the tissue culture research going on in our country and for young Hungarian research workers to get a glimpse of current international problems of tissue cultivation.

Experts of tissue culture methods were invited for the first time to Budapest on the occasion of the International Congress of Zoology in 1927. The first international meeting of experimental cytology was also part of this congress. The tissue culture technique which had already yielded valuable results was adopted for the first time here in Budapest by international representatives of the biological sciences. Tissue culture is today a widely accepted important method, the significance of which is duely reflected by the present Symposium.

We would like to express our gratitude for the support granted by the Hungarian Academy of Sciences enabling us to organize the 1971 Meeting of the European Tissue Culture Society and to present the material of the Symposium in this volume.

Our heartiest thanks are due to all researchers and organizers who have greatly contributed to the success of the Symposium. We are also indebted to the Publishing House of the Hungarian Academy of Sciences for the presentation of the proceedings.

The Editors

LIST OF PARTICIPANTS

Aros, B.
Department of Histology, Semmelweis Medical University, Budapest IX, Tűzoltó utca 58, Hungary

Astaldi, G.
The Blood Research Foundation Center, Municipal Hospital of Tortona, 15057 Tortona, Italy

Balázs, A.
Institute of Experimental Medicine, Budapest VIII, Szigony utca 43, Hungary

Bánóczy, J.
Institute of Oral and Maxillo-facial Surgery, Semmelweis Medical University, Budapest VIII, Mária utca 52, Hungary

Béládi, I.
Institute of Microbiology, Medical University, Szeged, Dóm tér 10, Hungary

Benczur, M.
Department for Transplantation Immunology, National Blood Center, Budapest XI, Daróczi út 24, Hungary

Blazsek, I.
Institute of Experimental Medicine, Budapest VIII, Szigony utca 43, Hungary

Boll, I.
Städtisches Krankenhaus Neukölln, I. Innere Abteilung, Rudower Strasse 56, 1 Berlin 47, Berlin-West

Buffe, D.
Institut de Recherches Scientifiques sur le Cancer, 16 Avenue-Vaillant-Couturier, Villejuif (Seine), France

Bukulya, B.
Institute of Experimental Medicine, Budapest VIII, Szigony utca 43, Hungary

Chèvremont, M. J. J.
Institut d'Histologie et d'Embryologie, Faculté de Médecine, Université de Liège, 20 rue de Pitteurs, 4000 Liège, Belgium

Csaba, Gy.
Department of Histology, Semmelweis Medical University, Budapest IX, Tűzoltó utca 58, Hungary

Dallman, L.
Institute of Genetics, József Attila University, Szeged, Ady tér, Hungary

Drén, Cs.
Veterinary Medical Research Institute, Hungarian Academy of Sciences, Budapest XIV, Hungária krt 21, Hungary
Fazekas, I.
Institute of Experimental Medicine, Budapest VIII, Szigony utca 43, Hungary
Fjelde, A.
Roswell Park Memorial Institute, Buffalo, New York 14203, U.S.A.
Franks, L. M.
Department of Cellular Pathology, Imperial Cancer Research Fund, Lincoln's Inn Fields, London, WC2A 3PX, United Kingdom
Gaillard, P. J.
Laboratorium voor Celbiologie en Histologie, Rijnsburgerweg 10, Leiden, The Netherlands
Gazsó, L.
Institute of Neurosurgery, Budapest XIV, Amerikai út 57, Hungary
Gross, W. O.
Institut d'Histologie, Université de Lausanne, 9 rue du Bugnon, CH 1005 Lausanne, Switzerland
Gyévai, A.
Institute of Experimental Medicine, Budapest VIII, Szigony utca 43, Hungary
Hilwig, I.
Farbwerke Hoechst AG, Gewebezüchtung H. 811, Johannesallee 10, 623 Frankfurt/Main–Höchst, German Federal Republic
Holečková, E.
Institute of Physiology, Czechoslovak Academy of Sciences, Praha 4, Krč, Budějovická 1083, Czechoslovakia
Horváth, L.
National Institute of Public Health, Budapest IX, Gyáli út 2, Hungary
Jordanov, J.
Institute of Morphology, Bulgarian Academy of Sciences, Boul. Lenin 75, bl. 103, Sofia, Bulgaria
Kahri, A.
2nd Department of Pathology, University of Helsinki, Hartmaninkatu 3, Helsinki, Finland
Kapa, E.
Department of Histology, Semmelweis Medical University, Budapest IX, Tűzoltó utca 58, Hungary
Kellermayer, M.
Central Clinical Laboratory, Medical University, Pécs, Ifjúság útja 31, Hungary
Kieler, J.
Fibiger Laboratory, Lundtoftevej 5, Kgs. Lyngby, Copenhagen, Denmark
Kiss, J.
Department of Histology, Semmelweis Medical University, Budapest IX, Tűzoltó utca 58, Hungary
Kiszely, Gy.
Biological Institute of Medical University, Szeged, Kossuth L. sgt 35, Hungary

KLEIN, J. C.
Radiobiological Institute TNO, Lange Kleiweg 151, Rijswijk Z. H., The Netherlands
KORITSÁNSZKY, S.
Department of Histology, Semmelweis Medical University, Budapest IX, Tűzoltó utca 58, Hungary
KÓSA, Zs.
National Institute of Public Health, Budapest IX, Gyáli út 2, Hungary
KOVÁCS, E. I.
Department of Evolution and Genetics, Eötvös Loránd University, Budapest VIII, Múzeum krt 4/a, Hungary
LUND, E.
Department of Veterinary Virology and Immunology, The Royal Veterinary and Agricultural University of Copenhagen, 13, Bülowsvej, 1870 Copenhagen V, Denmark
MACIERA-COELHO, A.
Institut de Cancérologie et Immunogénétique, 14 Av. Paul Vaillant-Couturier, 94 — Villejuif, France
MAREEL, M.
Kliniek voor Radiotherapie en Kerngeneeskunde, De Pintelaan 115, Gent, Belgium
MARÓTI, M.
Department of Plant Physiology, Eötvös Loránd University, Budapest VIII, Múzeum krt 4/a, Hungary
MATAGNE-DHOOSSCHE, F.
Institut d'Anatomie, Laboratoire d'Histologie, rue de Pitteurs, 4000 Liège, Belgium
MICHL, J.
Institute of Physiology, Czechoslovak Academy of Sciences, Praha 4, Krč, Budějovická 1083, Czechoslovakia
NEMESKÉRI, A.
Department of Histology, Semmelweis Medical University, Budapest IX, Tűzoltó utca 58, Hungary
NEUPERT, G.
Pathologisches Institut der Friedrich-Schiller-Universität, Ziegelmühlenweg 1, 69 Jena, German Democratic Republic
OLÁH, E.
Research Institute of Oncopathology, Budapest XII, Ráth György utca 7, Hungary
OLIVO, O. M.
Istituto di Anatomia, Via Irnerio, 48, 40126 Bologna, Italy
PÁLYI, I.
Research Institute of Oncopathology, Budapest XII, Ráth György utca 7, Hungary
PIECK, A. C. M.
Laboratory of Chemical Cytology, Driehuizerweg 200, Nijmegen, The Netherlands
RAPPAY, Gy.
Institute of Experimental Medicine, Budapest VIII, Szigony utca 43, Hungary

de RIDDER, L.
Kliniek voor Radiotherapie en Kerngeneeskunde, De Pintelaan 115, Gent, Belgium

ROUSSEAU, M. F.
Laboratoire d'Anatomie Pathologique, Inserm U 77, Groupe Hospitalier Necker–Enfants Malades, 149, rue le Sèvres, Paris 15e, France

RÖHLICH, P.
1st Central Laboratory of Electron Microscopy, Department of Histology, Semmelweis Medical University, Budapest IX, Tűzoltó utca 58, Hungary

RUZICSKA, P.
National Institute of Public Health, Budapest IX, Gyáli út 2, Hungary

SCHERFT, J. P.
Laboratory for Cell Biology and Histology, University of Leiden, Academic Hospital, Rijnsburgerweg 10, Leiden, The Netherlands

SCHLEICH, A.
Institut für Experimentelle Krebsforschung der Universität Heidelberg, Voss-Str. 3, 69 Heidelberg, German Federal Republic

SCHWÖBEL, W.
Bundesforschungsanstalt für Viruskrankheiten der Tiere, Paul-Ehrlich-Strasse 28, 74 Tübingen, German Federal Republic

SELLYEI, M.
Róbert Károly Hospital, Department of Pathology, Budapest XIII., Róbert Károly krt 82, Hungary

SPURNÁ, V.
Institute of Biophysics, Czechoslovak Academy of Sciences, Brno 12, Královopolská 135, Czechoslovakia

STARK, E.
Institute of Experimental Medicine, Budapest VIII, Szigony utca 43, Hungary

STÄHELIN, H.
Sandoz AG, 4002 Basel, Switzerland

STREET, H. E.
School of Biology, University of Leicester, Adrian Building, University Road, Leicester, LE1 7RH, United Kingdom

TANNEBERGER, ST.
Institute für Medizin und Biologie, Robert-Rössle-Klinik, Deutsche Akademie der Wissenschaften zu Berlin, Lindenberg Weg 80, 1115 Berlin-Buch, German Democratic Republic

TÉTÉNYI, P.
Research Institute of Medicinal Plants, Budapest XII, Dániel út 40, Hungary

THUST, R.
Pathologisches Institut der Medizinischen Akademie, Nordhäuser Str. 74, 50 Erfurt, German Democratic Republic

TIXIER-VIDAL, A.
Laboratoire de Biologie Moléculaire, Collège de France, 11, Place Marcelin-Berthelot, Paris–Ve, France

TÖRŐ, I.
Department of Histology, Semmelweis Medical University, Budapest

IX, Tűzoltó utca 58, and Institute of Experimental Medicine, Budapest VIII, Szigony utca 43, Hungary

TÖRÖK, O.
Department of Histology, Semmelweis Medical University, Budapest IX, Tűzoltó utca 58, Hungary

TYIHÁK, E.
Research Institute of Medicinal Plants, Budapest XII, Dániel út 40, Hungary

VÁGÚJFALVI, D.
Research Institute of Medicinal Plants, Budapest XII, Dániel út 40, Hungary

VALENTINI, A. F.
Istituto di Anatomia, Via Irnerio, 48, 40126 Bologna, Italy

VERNE, C.–M. J.
Institut d'Histochimie Médicale, Faculté de Médicine de Paris, 45 rue des Saints-Pères, Paris VI^{e}, France

VETTER, J.
Department of Plant Physiology, Eötvös Loránd University, Budapest VIII, Múzeum krt 4/a, Hungary

WALLER, M.
Pathologisches Institut, Martin-Luther-Universität, Halle–Wittenberg, Leninallee 14, 402 Halle (Saale), German Democratic Republic

WOLLEMANN, M.
Institute of Neurosurgery, Budapest XIV, Amerikai út 57, Hungary

LIST OF CONTRIBUTORS

Bácsy, E.
Institute of Experimental Medicine, Budapest, Hungary

Balázs, A.
Institute of Experimental Medicine, Budapest, Hungary

Blazsek, I.
Institute of Experimental Medicine, Budapest, Hungary

Bukulya, B.
Institute of Experimental Medicine, Budapest, Hungary

Davey, M. R.
Department of Botany, University of Nottingham, Nottingham, United Kingdom

Fazekas, I.
Institute of Experimental Medicine, Budapest, Hungary

Fjelde, A.
Roswell Park Memorial Institute, Buffalo, N.Y., U.S.A.

Franks, L. M.
Department of Cellular Pathology, Imperial Cancer Research Fund, London, United Kingdom

Gross, W. O.
Histologisch-Embryologisches Institut der Universität Lausanne, Lausanne, Switzerland

Gyévai, A.
Institute of Experimental Medicine, Budapest, Hungary

Jobst, K.
Central Clinical Laboratory, Medical University, Pécs, Hungary

Kahri, A. I.
2nd Department of Pathology, University of Helsinki, Helsinki, Finland

Kellermayer, M.
Central Clinical Laboratory, Medical University, Pécs, Hungary

Lyytikäinen, A.
University of Helsinki, Helsinki, Finland

Mihály, K.
Institute of Experimental Medicine, Budapest, Hungary

Ökrös, I.
Institute of Experimental Medicine, Budapest, Hungary

Pesonen, S.
University of Helsinki, Helsinki, Finland

Rappay, Gy.
Institute of Experimental Medicine, Budapest, Hungary

Röhlich, P.
1st Central Laboratory of Electron Microscopy, Semmelweis Medical University, Budapest, Hungary
Saure, A.
University of Helsinki, Helsinki, Finland
Stark, E.
Institute of Experimental Medicine, Budapest, Hungary
Street, H. E.
Botanical Laboratories, University of Leicester, Leicester, United Kingdom
Sutton-Jones, B.
School of Biological Sciences, University of East Anglia, Norwich, United Kingdom
Szalay, K.
Institute of Experimental Medicine, Budapest, Hungary
Tixier-Vidal, A.
Laboratoire de Biologie Moléculaire, Collège de France, Paris, France
Törő, I.
Department of Histology and Embryology, Semmelweis Medical University, Budapest, Hungary
Török, O.
Department of Histology and Embryology, Semmelweis Medical University, Budapest, Hungary

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WELCOMING ADDRESS

by

I. TÖRŐ

PRESIDENT OF THE YEAR OF THE EUROPEAN TISSUE CULTURE SOCIETY

Ladies and Gentlemen,

I am gratified by the honour to be able to welcome you in Budapest at the 1971 Session of the European Tissue Culture Society. Pleasant memories of bygone meetings come to my mind and of the delightful days we spent in various countries where we assembled.

In the succession of these meetings the progress of tissue culture, its evolution as a branch of science is well reflected. At the outset the main object was the observation and study of the behaviour of explanted tissues and cells, but later morphological and structural changes and also differences in cell potentials were extensively investigated. By studying the prerequisites of cell differentiation and dedifferentiation several valuable results have been obtained throwing light on the organisatory forces manifesting in tissues removed from the influence of the organism and placed into changed circumstances. What changes are manifested *in vitro*, which refer to differences observed *in vivo* and which are the histological and cytological phenomena in cultures which by absence reveal the organisatory forces of the organism. Pure homogeneous cultures and their confrontation have cleared the way to the understanding of such organisatory forces which are based on the mutual influences of different tissues and could give information for the analysis of early embryonal development.

It has been observed, namely, that extracellular factors dominating in cultures cause intracellular alterations which, in turn, affect the homeostasis of the tissues. These observations have raised the idea that cell proliferation which dominates in *in vitro* processes and concomitant nucleic acid synthesis will inhibit the synthesis of specific proteins, i.e. dedifferentiation will bring a change also in cell potentials. The extent to which dedifferentiation of the cell of higher animals may serve to provide precursor cells is not known. It has generally been assumed that genome repression in dedifferentiated cells of vertebrates is irreversible, however, such repression in highly differentiated cells can be abated under certain experimental conditions. The state of genome repression in higher animals as well as in simple organisms, depends on environmental conditions both within and outside the cell. Differentiation can be reversed under appropriate circumstances.

We surmise that genomes suppressed during differentiation can get released during cultivation and together with the structural changes let the pluripotence of cells become manifested.

For this reason we believe it would not be without merit to devote the first day of our Conference to the results of ultrastructural investigations of some cultures.

Symp. Biol. Hung. 14, pp. 21–29 (1972)

FINE-STRUCTURAL DIFFERENTIATION OF LYMPHORETICULAR CELLS IN CULTURE

by

I. Törő, I. Fazekas, I. Ökrös, E. Bácsy, and Gy. Rappay
INSTITUTE OF EXPERIMENTAL MEDICINE, BUDAPEST, HUNGARY

The changed attitude prevailing in recent years in the research on cells cultivated *in vitro* may be explained by two reasons. One is that following the early experimental phase of tissue culture techniques it is today attempted to define consciously the interrelation between explant and medium, i.e. not only to keep the explant alive for a shorter or longer period but also to make the *in vitro* cultivated cells function normally by choosing the environment properly. The studies aimed at the demonstration of the function of endocrine cells *in vitro* are a most impressive example of this trend (Stark et al., 1965*a*, *b*; Kowal, 1970; Sato et al., 1970).On the other hand, modern more effective methods like electrophysiology, autoradiography, electron microscopy, etc. have become available for studying structural changes in the cells during cultivation for shorter or longer periods. This enables us to compare the results of cytophysiology and cytomorphology and to draw conclusions of molecular-biological importance. Although the study of the fine structure of cells cultivated *in vitro* has gained ground, in most of the cases the findings have not been compared with those gained *in vivo*. Such a comparison might supply answers to fundamental cyto-

TABLE 1

Cell types present *in vivo*[*]

Thymocytes	Reticulum cells			Other cells
large[b] medium[b] small[b]	epithelial cytoreticulum[a1] ↓ reticular mainly in the cortex	↓ hypertrophic mainly in the medulla	mesenchymal[a2] ↓ macrophages ↓ special histiocytic cell (van Haelst)	fibrocytes[a3] mast cells granulocytes plasma cells (?) endothelial cells[a1]
many mitotic figures	no mitosis			no mitosis

Cell types present *in vitro* after 4-day culturing

epithelial-like cells[a1]
fibroblast-like cells[b]
true fibroblasts[a3]
macrophages[a2b]
} many of them mitotic

* Symbols *a* and *b* indicate possible transformations.

logical questions. Other studies in the present volume will be found to be good examples of the change in attitude.

There are a number of interesting questions awaiting answer in the field of tissue cultivation: does the fine structure of cultivated cells change as compared with the original cells, and if so, to what extent; which cytological characteristics disappear and which persist during cultivation? The most important question probably is how any such change will influence the original specific function of the cells.

The same questions arose in the course of our fine-structural and enzyme-cytochemical analysis of thymus explants.

The thymus is rich in thymocytes and reticulum cells and contains various other cells, too (Table 1). The reticulum cells are of mesenchymal or entodermal origin. Those derived from the entoderm form a peculiar network, the cytoreticulum. They are easily recognizable by their desmo-

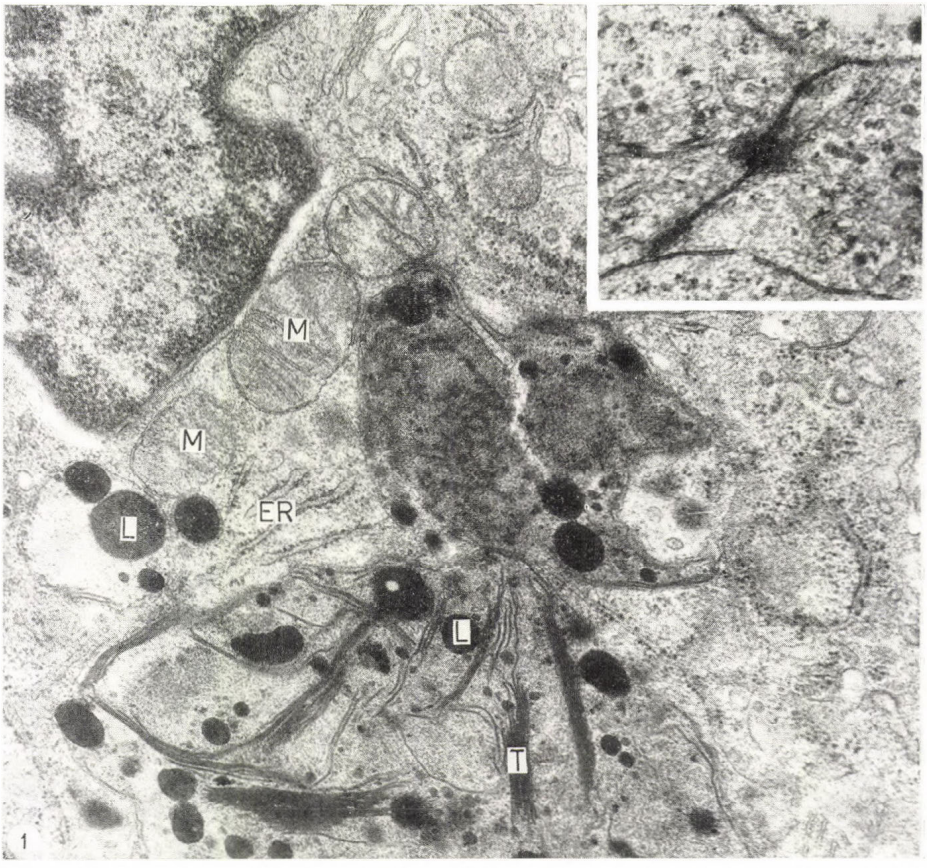

Fig. 1. Portion of an epithelial reticular cell from intact rat thymus. Tonofilaments (T), primary lysosomes (L), cristate mitochondria (M), rough-surfaced endoplasmic reticulum (ER), granules of different density in the cytoplasm. ×44,500. Inset: A typical desmosome characteristic of the epithelial reticular cells. (Glutaraldehyde-osmium fixation; uranyl acetate and lead citrate staining) ×32,700

somes, tonofilaments and special vacuoles (Fig.1). Their specific function is to form the supporting network, further endocytosis and, in our and other authors' opinion, the production of the lymphocyte-stimulating factor(s) (Clark, 1966, 1968; Ito, 1966; Gad and Clark, 1968; Hirokawa, 1969; Goldstein and White, 1970; Goldstein et al., 1970; Rappay et al., 1971). The most important sign of this last-named function is the presence of special vacoules in the cytoplasm, empty or densely packed.

Rat thymus fragments were explanted on top of coagulated chicken plasma and chick-embryo juice on cover-slips. Each slide, with 5 fragments on it, was then placed into a Leighton tube, in which it was allowed to stand for 24 h. After this time, 2 ml of a medium consisting of an 8 : 2 mixture of TCM 199 and heat-inactivated calf serum, with 200 I.U. of penicillin added to it per ml, was poured into each tube. The culture medium was exchanged every other day. Cultures were fixed at different time intervals in the period between the 4th and the 30th day after explantation. Cultures together with the glass slides were fixed for electron microscopy in 4·5 per cent glutaraldehyde diluted with 3·13 M cacodylate buffer at pH 7 for periods of 30 to 120 min, and then washed with the diluent buffer for 1 to 16 h depending on the concentration of the fixative and the length of fixation time. Subsequently, the cultures were either postfixed for 30 to 90 min with 1 per cent osmium tetroxide dissolved in cacodylate buffer and embedded in Durcupan ACM, by using propylene oxide, or histochemically analysed (Table 2).

TABLE 2

Cytochemical reactions for studying thymus explants

Tests for	Cryostat sections and cultures	
	LM	EM
Acid phosphatase	Naphtol AS-BI or TR phosphate	Sodium-β-glycerophosphate
	Freshly diazotized 5-chloro-2-toluidine	Lead nitrate
	Michaelis veronal-acetate buffer, pH 5.0	0.05 M acetate buffer, pH 5.2
	Incubation time: 30–60 min	Incubation time: 1h
Non-specific esterase	Naphtol AS-D acetate	Thioacetic acid
	Fast Blue BB	Lead nitrate
	0·1 M tris-maleate buffer, pH 7·1	0·05 M cacodylate buffer, pH 5·5
	Incubation time: 2–5 min	Incubation time: 30–60 min (+4 °C)

In the growth zone of the thymus explants (Fig. 2) the desmosomes and tonofilaments help to identify the epithelial reticulum cells even after 21 days of cultivation (Fig. 3). The special vacuoles presumably involved in the production of the lymphocyte-stimulating factor(s) were not seen either early or later during cultivation (4–30 days), suggesting that thymic epithelial reticular cells do not produce lymphocyte-stimulating hormone *in vitro*. Considering that no thymocytes were present in these cultures, it seems that the presence of these cells is a precondition for the production

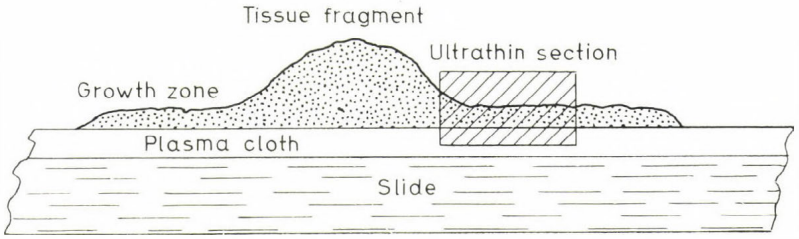

Fig. 2. Scheme of the culture method and of the preparation of ultrathin sections

Fig. 3. Portions of two epithelial reticular cells from a 21-day-old culture with a desmosome (D) and tonofilaments (T). The cytoplasms are relatively poor in organelles. (Glutaraldehyde-osmium fixation; uranyl acetate and lead citrate staining) ×34,000

of the lymphocyte-stimulating factor(s). Consequently, one of the assumed specific funtions of epithelial reticular cells is not realized *in vitro*.

Endocytosis is a specific function of the epithelial, and also of the mesenchymal reticulum cells in the intact thymus; it is connected with the lysosomes present in the cytoplasm. In the intact thymus the reticulum cells of different origin can be differentiated on the basis of their enzyme activity

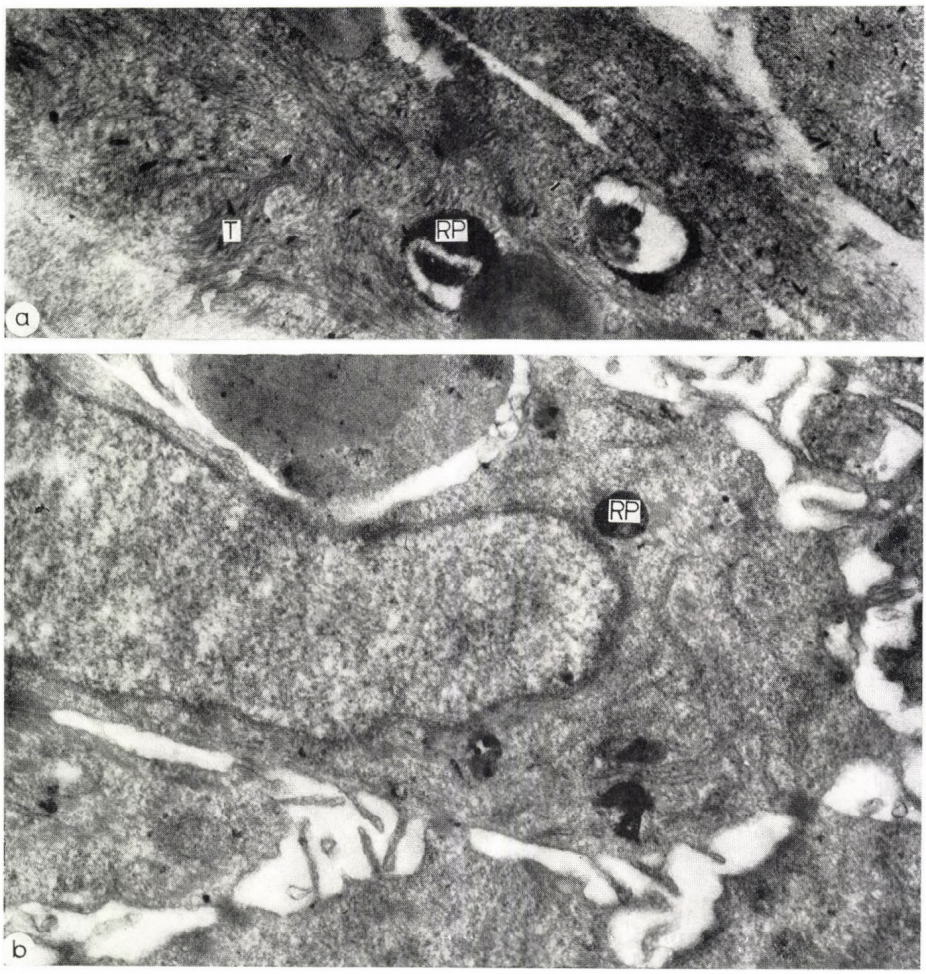

Fig. 4. Acid phosphatase (*a*) and non-specific esterase (*b*) activity in epithelial reticular cells. (*a*) 21-day-old culture. Note reaction product (RP) in large lysosomes. The portion of the cytoplasm is rich in tonofilaments (T) and in other cell organelles. ×44,000. (*b*) 15-day-old culture. Reaction product (RP) is observable in dense bodies (lysosomes). (Glutaraldehyde-osmium fixation; uranyl acetate staining) ×27,400

(Ökrös et al., 1969*a*, *b*). The lysosomes of the epithelial reticulum cells show a moderate acid phosphatase activity, and a considerably lower non-specific esterase activity (Fig. 4). In the mesenchymal reticulum cells both enzymes are more active than in the epithelial cells. In the thymic explants, considerable acid phosphatase activity and non-specific esterase activity were shown only by the mesenchymal epithelial cells, i.e. macrophages (Fig. 5). This did not show any correlation with the time of cultivation. Thus the endocytotic activity of the epithelial reticulum cells also seemed to have decreased in our culture, whereas the mesenchymal reticulum cells had preserved their intensive macrophage function. The formation

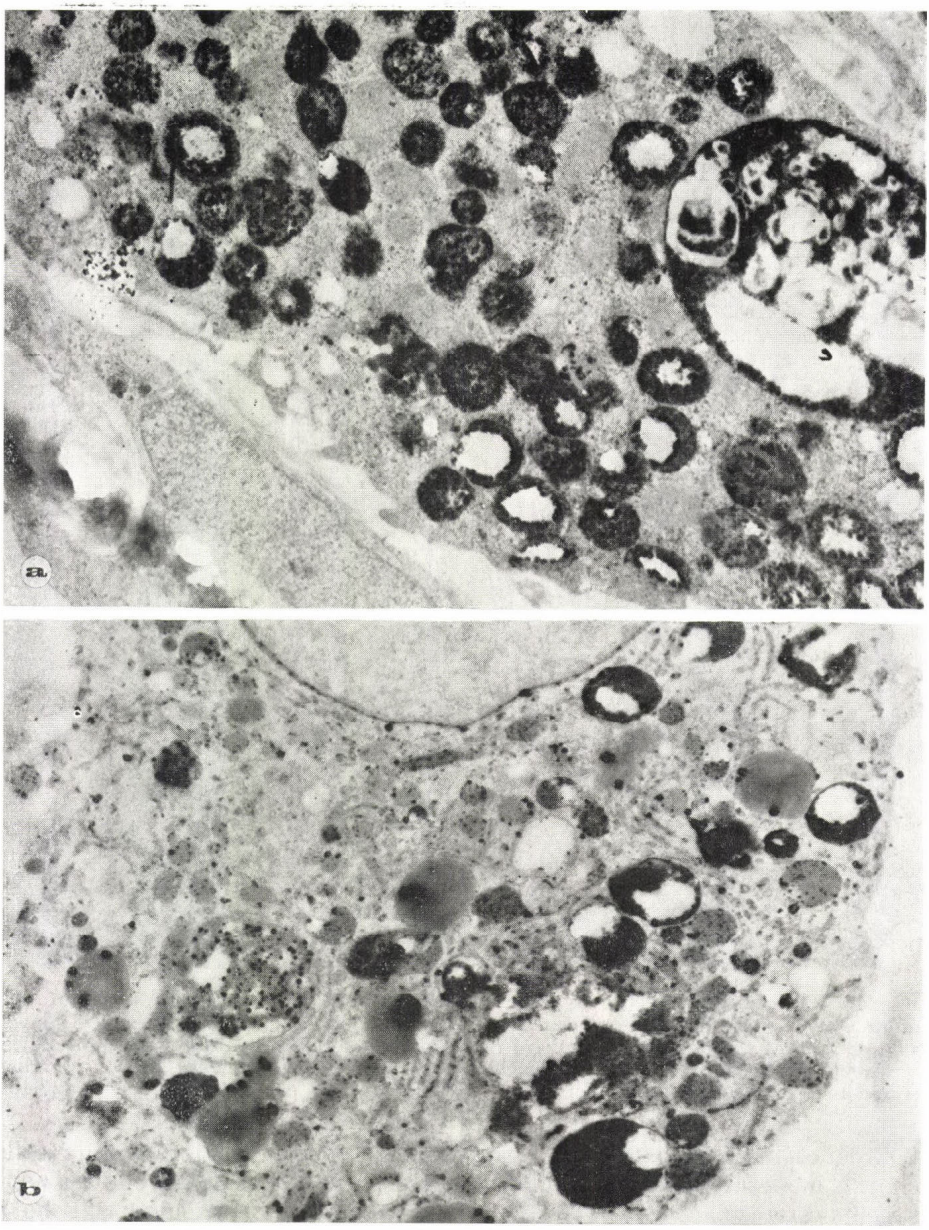

Fig. 5. Acid phosphatase (*a*) and non-specific esterase (*b*) activity in mesenchymal reticular cells (macrophages). (*a*) 21-day-old culture. The cytoplasm contains a great number of primary and secondary lysosomes with intense enzymic activity. $\times 16{,}000$. (*b*) 6-day-old culture. Esterase activity is localized mainly in primary and secondary lysosomes, and lipid droplets; a moderate enzymic activity presents itself in nuclear envelope, endoplasmic reticulum and some mitochondria. (Glutaraldehyde-osmium fixation; uranyl acetate and lead citrate staining) $\times 13{,}400$

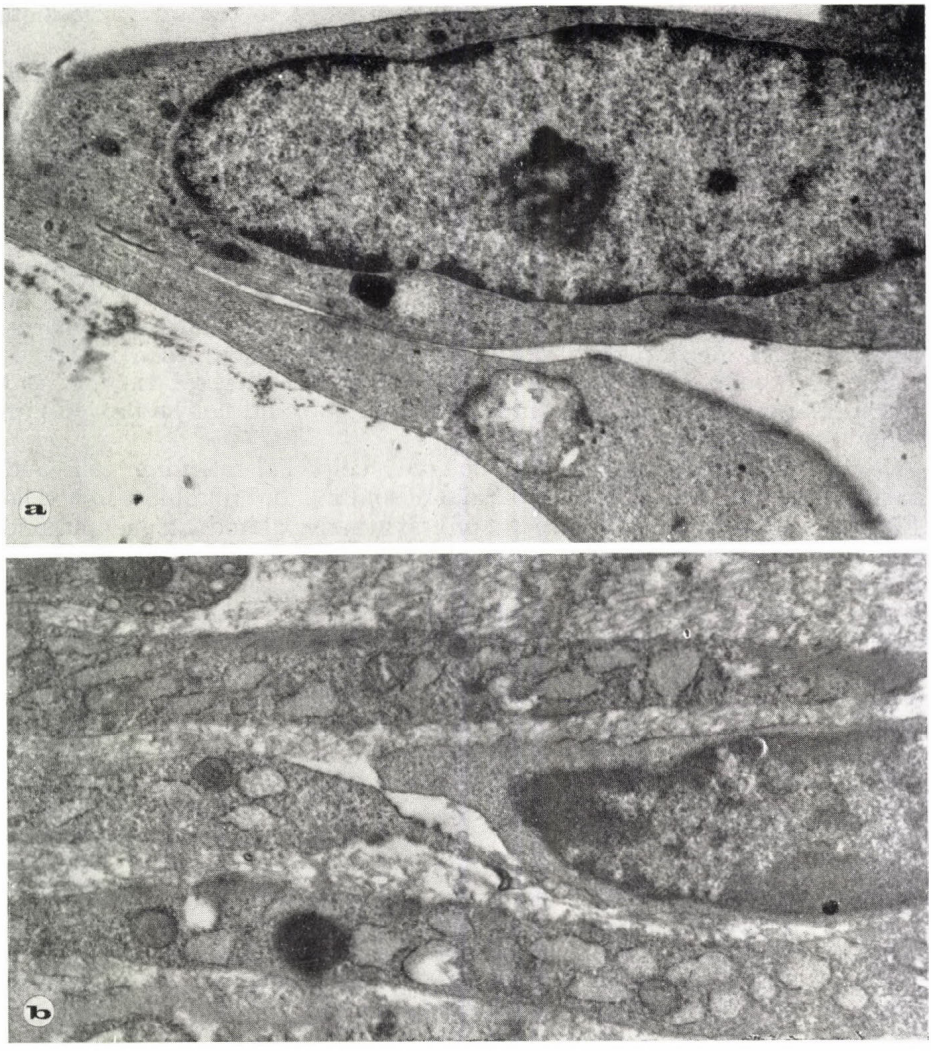

Fig. 6. Fibroblast-like cells (*a*) and true fibroblasts (*b*). (*a*) 19-day-old culture. Portions of fibroblast-like cells characterized by a cytoplasm poor in organelles. There is no sign of collagen fibre formation. × 19,400. (*b*) 9-day-old culture. Cytoplasms showing well-developed and dilated endoplasmic reticulum. Note the relatively dense material in cisternae of the endoplasmic reticulum and signs of fibre formation. × 20,000

of the supporting network was, therefore, the only function of the epithelial reticulum cells the morphological signs of which could still be demonstrated *in vitro*.

In vitro conditions not only influence the specific functions of the cells but also change the cellular composition of the original explant. The change in the incidence of different cell types is due to the declined viability of some cell types, to the altered proliferation rate of non-parenchymal cells,

and of the surviving parenchymal cells, as well as to the transformation of some of the cells originally present in the explant.

In the adult rat thymus only the thymocyte precursors are capable of mitosis (Table 1). In thymus explants, on the other hand, the number of mitotic cells is relatively great, but their identification is difficult. The number of epithelial reticulum cells increases parallel with the time of cultivation, because these cells regain their mitotic ability in culture, i.e. their autosynthetic function is realized *in vitro*. As a matter of fact, their nuclei contain little heterochromatin, and one or two marked nucleoli. There is also an increase in the number of macrophages. This may be explained with the mesenchymal reticulum cells becoming capable of mitosis or with the transformation of the cells of the original explant into macrophages (Nelson, 1969). According to several authors, lymphocytes can be transformed into macrophages (Nelson, 1969); in our material the *in vitro* transformation of lymphocytes into macrophages could not be proved but it could not be excluded either.

Our thymus explants contained not only epithelial reticulum cells and macrophages but also a number of true fibroblasts and fibroblast-like cells (Fig. 6). The signs of intensive protein synthesis: abundant endoplasmic reticulum, the appearance of dilated saccules and cysternae, and the presence of collagenous fibres, all point to their original specific function having been preserved. Of the functions of the fibroblast-like cells whose cytoplasm is poor in organelles, only autoreproduction was evident.

The living organism as well as the individual organs are controlled by general and specific regulatory systems (Goss, 1967). In the case of isolated cells *in vitro* there is no such complex regulation. The regulatory factors whose presence is needed in the artificial media to maintain the specific function of the explanted cells for a well-defined period, or infinitely, could be reliably identified only for a limited number of cells. A more penetrating insight into the factors influencing the specific functions and autosynthetic activity of the cells of lymphoreticular organs in *in vitro* cultures would help considerably in the understanding of the regulatory mechanism functioning *in vivo*. This requires, in addition to functional investigations, the knowledge of the fine structure of the cells. Further fine-structural investigations on cultivated thymic cells might significantly contribute to our knowledge of the functional mechanisms of the thymus.

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THE ULTRASTRUCTURE OF TISSUE CULTURE CELLS

by

L. M. Franks
DEPARTMENT OF CELLULAR PATHOLOGY, IMPERIAL CANCER RESEARCH FUND, LONDON, UNITED KINGDOM

The problem of identity is a major preoccupation for philosophers in the 20th century and my talk today concerns itself with a small facet of this central problem of our civilization. It underlines the uncertainties of an identity which depends only on appearances, particularly when those appearances are shadows. Morphology is a useful guide to identity but, as in so many other cases, it is the pattern which is as important as the detailed structure of the individual components. Thus morphology will allow us to identify with certainty most tissues but the identification of an individual cell from any given tissue may not be possible unless it has some clearly recognisable marker. This is the central problem for those concerned with the ultrastructure of tissue culture cells.

THE ULTRASTRUCTURE OF MOUSE CELL LINES

I am going to describe the ultrastructure of a number of cell lines derived from normal tissues and from tumours and try to draw some conclusions from our findings. The first experiment I am going to describe was set up to compare the growth potential of young and old tissues (Franks and Henzell, 1970). Since there is an increasing incidence of tumours in some organs with age our original premise was that if there was a cellular basis for this increased neoplastic potential, cells from old organs should undergo spontaneous neoplastic transformation more readily than cells from young tissues. Thirty-six cell lines were established from various organs of embryo (18 day), young (3–20 days) and old (28–34 months) C3H and C57BL(a^t) mice. Spontaneous neoplastic change occurred in 16 of these lines, but there was no specific age relationship. The ultrastructure of 11 tumour lines and 13 non-tumour lines were studied at different transfer generations by transmission electron microscopy (Franks and Wilson, 1970) and some by scanning electron microscopy (Hodges, 1970). The cells in all cultures were similar whatever the organ from which they were derived (kidney, lung, bladder, tongue, heart, spleen, prostate, peritoneum and spinal cord).

We have described the ultrastructure of these cells in detail in an earlier paper (Franks and Wilson, 1970). There were 2 main types of cell. Type 1 cells had rounded or bean-shaped nuclei with a thin rim of marginal chromatin, and the cytoplasm contained some rough endoplasmic reticulum, many free ribosomes, a small Golgi zone and relatively few lysosomes, autophagic vacuoles and mitochondria. Cell processes were short and few in number.

Type 2 cells had a more convoluted nucleus in which the peripheral chromatin layer was thicker and small clumps of chromatin were scattered throughout the nuclear matrix. The Golgi zone was large; there were usually many lysosomes and autophagic vacuoles and often dilated cisternae of rough endoplasmic reticulum. The cells were sometimes rounded but more usually the surface was very irregular with long sheet-like cytoplasmic fringes extending for a considerable distance from the cells. In suspensions prepared by scraping these processes were oriented along a layer of extracellular fibrillar material and often interdigitated with similar processes from other cells. Although most cells could easily be classified as type 1 or 2 there were some atypical cells in most cultures. Most of these had the cytoplasmic characters of type 1 cells but the nuclei were more convoluted. Other cells had more cytoplasmic organelles than the typical type 1 cells. This suggests that there may be a transition between type 1 and type 2 cells. Occasional giant cells were also present. Only their size distinguished them from other type 1 and type 2 cells. There were also a number of other ultrastructural features. Most of the cells had intracellular actin-like filaments (Franks et al., 1969), sometimes in very large numbers. Micropinocytotic vesicles were very frequent in the peripheral areas of cells, especially in those with many filaments. Specialized cell contacts were present between all cell types mostly intermediate junctions (zonula adherens) but a few atypical tight junctions (zonula occludens) were also found. Thickenings of the inner aspect of the plasma membrane, resembling attachment bodies of smooth muscle, were often found. These were usually associated with extracellular material resembling basal lamina. The distribution of this basal lamina-like material is best seen in preparations in which the cells are fixed and embedded *in situ*, without removal from the glass on which they have grown. Using a technique developed by my colleague, Mr. Cooper, it can be seen that this material is probably also concerned with the attachment of cells to glass. Most of the other extracellular material was not collagen. It had 2 components, an amorphous material and fine tubular fibrils about 100Å in diameter, resembling the fibrillar component of elastic tissue.

The surface structure of some of these cells had been described by Dr. Gisele Hodges (1970). When the cell lines which have undergone neoplastic transformation are inoculated into syngeneic mice they all produce similar tumours, whatever the organ of origin (Franks et al., 1970).

THE ULTRASTRUCTURE OF CELL LINES FROM OTHER SPECIES

These findings raised two questions. Firstly, are there in fact 2 fixed cell types or can one type change into the other? Secondly, where do the tissue culture cells come from? To answer the first question we established 8 clones from one cell line. Two of these contained both cell types, one contained type 2 cells only and 5 contained only type 1 cells. The two mixed 'clones' may not have been truly cloned so that it seems that two types may be stable but we cannot yet exclude the possibility of a transition. We hope to answer this question shortly.

The second question is more difficult to answer. One possibility is that our culture conditions do not allow the survival of differentiated parenchymal cells from the organs we have cultured and that we have selected out the same 'undifferentiated' cells from each organ. The other possibility is that the tissue culture cells are in fact derived from specific parenchymal cells but undergo a structural modulation in culture, so that they all have a similar appearance. There are few detailed descriptions of the ultrastructure of cell lines derived from normal tissues. We, therefore, made a brief survey of a number of cell lines established in other laboratories, and from other species than the mouse. These included the Balb/c 3T3 line established by Aaronson and Todaro (1968), NIL cells and secondary hamster embryo cultures (all provided by Dr. Ian McPherson) and a line derived from rat liver by Dr. Kaigh (provided by Dr. B. Weinstein). These cell lines have an ultrastructure similar to that we have described in our mouse cell lines.

Finally we have examined cells from many lines derived in our laboratories from human embryos (Franks and Cooper, in preparation). In lines derived from lung, the ultrastructure is similar to that I have described, not only in cell types but in the distribution of extra-cellular basal lamina-like material most easily seen in cells sectioned *in situ*.

Again, there are few detailed descriptions of the ultrastructure of human embryo cell lines but we were encouraged to find a description of the ultrastructure of cells derived from human skin cultures (Comings and Okada, 1970) which appear to be identical to those we have described.

THE POSSIBLE NATURE OF TISSUE CULTURE CELLS

In primary explant cultures or in organ cultures, specific epithelial cells can be recognized at the light microscope and ultrastructural levels (e.g. Franks and Barton, 1960) but, as has been recognized for many years, differentiated cells in culture are usually 'overgrown' by so-called fibroblasts. If there is selection of cells rather than a structural change affecting differentiated cells, the cells which are eventually selected should be present in the initial cell suspensions or explants from which the cultures were established. We, therefore, examined primary cell suspensions and partially trypsinized tissue fragments to see if we could identify cells similar to those which eventually predominate in culture.

In suspensions prepared from mouse kidney (Franks and Wilson, 1970) cells resembling type 1 and type 2 cells can be seen associated with the glomerular capillaries and attached to the outer walls of some tubules. In preparations from human embryo lung the possible site of origin of the cells can be seen more clearly. The histology of the human embryo lung has been described by Cooper (1938) and the appearances in primary cultures by Chesterman and Franks (1960). The epithelial cells have a distinctive appearance quite different from that of the cells which are found in the later cultures. In the stroma groups of mesenchymal cells can be seen. Some are fibroblasts and others have many of the features seen in the type 2 tissue culture cells, i.e. irregular convoluted nuclei, intracellular fibrils, often in large numbers, peripheral micropinocytotic vesicles, specialized cell contacts, etc. Many of these cells are in solid cords, but others surround

a central lumen. In some cases cells resembling type 1 and 2 cells can be seen forming the walls of small capillaries and flattened type 2 cells with many microvilli are sometimes found lining larger venules. Thus one can establish a morphological identity and probable vascular origin for the tissue culture cells but we have no other method for positive identification. We have discussed in an earlier paper (Franks and Wilson, 1970) the evidence suggesting that these cells may be derived from endothelial cells and pericytes, but we cannot yet exclude the possibility that these cells may still be derived from specific parenchymal cells but have lost any morphological markers.

THE ULTRASTRUCTURE OF TUMOUR CELLS

I have been talking so far only about cells derived from normal tissues. I am now going to describe briefly the ultrastructure of some cell lines derived from tumours. I am only going to discuss lines which have been established in our own laboratories, so that they have been grown under the same conditions as the 'normal' cell lines I have been talking about. Some of these tumour lines retain specific markers which allow their tissue of origin to be identified. The examples I have selected are a human bladder tumour line (Rigby and Franks, 1970) and 2 mouse mammary tumour lines (Franks and Rowlatt, 1968; Riddle 1971, in preparation). The bladder tumour has many of the morphological features of bladder epithelium and the cells produce a nodule of recognizable bladder tumour after heterotransplantation into the hamster cheek pouch. Two cell lines were established from one of the mammary tumours by Dr. Riddle. One of these formed alveolar structures in culture and was recognizably epithelial under the electron microscope. These cells produced a papillary mammary tumour when transplanted into syngeneic mice. The other line is similar in morphology to the cell lines derived from normal tissue and produces a similar type of tumour after transplantation. Dr. Riddle feels that this line is a result of a spontaneous neoplastic transformation of a cell line derived from normal stromal tissue present in the original tumour explants. The third example is even more confusing. This line is from a mammary tumour which arose in a male mouse (Franks and Rowlatt, 1968). This line is morphologically indistinguishable from our spontaneously transformed cell lines. Transplantation of the early culture generations produced a mixed tumour with some epithelial and some sarcomatous areas. Transplants of later culture generations only produce sarcomas similar to our transformed cell tumours.

CONCLUSIONS

Cell lines derived from some normal mouse, rat, hamster and human tissues have a similar ultrastructural morphology, whatever their tissue of origin. Tissue specific markers have not been found. The cells are morphologically similar to endothelial cells and pericytes but the possibility that the tissue culture cells may be derived from specific parenchymal cells, but have lost morphological markers, cannot be excluded. Some cell lines derived from

human and mouse tumours may still retain tissue specific morphological markers. In others, tissue specific cells may eventually be replaced by the same type of cell found in cultures derived from normal tissue. Morphology alone may not be a reliable method for establishing cell identity.

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THE ESTABLISHMENT IN CULTURE OF PERMANENT LINES OF HAEMATOPOIETIC CELLS, AND RECENT VIRUS STUDIES DONE WITH HAEMATOPOIETIC CELLS*

by

A. FJELDE
ROSWELL PARK MEMORIAL INSTITUTE, BUFFALO, N.Y., U.S.A.

An investigation of the period of establishment of (diploid) haematopoietic cells from normal individuals showed that the establishment of permanent human lines of haematopoietic cells from peripheral blood is not demonstrably dependent on structural or numerical changes at the chromosome level. Cultures from normal individuals have been examined by us at the time of establishment, and all had the normal diploid chromosomal complement of 46; aberrations were rarely seen.

At the time cell lines become established, there develops a surface attraction between the cells. They become loosely clumped in stationary cultures and, upon shaking, tend to reaggregate even after separation. After the initial lag period, the leukocytes, (routinely cultured in stationary flasks), begin to clump; it is at this stage that they also begin to grow rapidly. The interval between the time the cultures are initiated and the appearance of clumps and rapid growth ranged from 48 to 159 days in the cultures examined.

The cells in established cultures remain diploid and do not adhere to the surface of the glass. There is one exception: RPMI 8226, a human myeloma line which is triploid, the cells sometimes adhere to the glass surface and produce the typical myeloma protein. Electron-microscope studies revealed characteristic morphology for several types of cells propagated in culture.

The significant sequential events in the development of human haematopoietic cell lines from normal sources were: (*i*) a preliminary morphological transformation to stem cell and lymphoid types (before establishment); (*ii*) at the time of establishment, without PHA, growth and loose clumping of cells, which tend to reaggregate after shaking, indicative of a surface attraction; and (*iii*) after establishment, and 3 months of rapid growth, an apparently infinite life span (Fig 1). In a four-year-old myelogenous leukaemia line, cells showing slightly more or less than the diploid number of 46 chromosomes per cell, and marker chromosomes and aberrations usually associated with areas of secondary constriction, were evident. The cells from normal individuals in established cultures remained diploid. It is noteworthy that they did not adhere to glass surfaces, but instead adhered to each other.

Presumably, the established cell culture lines with infinite life spans are the products of self replicating cells, or cells which are able to divide

* Shortened form of the lecture.

and maintain a self-perpetuating population. The mechanisms by which cell populations become established *in vitro* are presently poorly understood. A morphological transformation and a deviation from the diploid chromosome pattern for the species have been thought to be associated with cell proliferation; however, in our studies the significant event associated with a typical sudden initiation of rapid growth was a tendency for the cells to aggregate.

The dynamics of growth effects can be observed in cultures during this period of establishment. The following charts show the results obtained during the initial phase of growth. Table 1 shows a typical experiment with peripheral leukocytes from normal donors illustrating the diploid stemline characteristic of cells at the time of establishment. All of the cultures showed a normal value of less than two per cent chromosomal aberrations. Morphological examination revealed transformed and normal appearing lymphocytes such as those seen in three-day PHA-treated cultures. According to Table 2 more than 75 per cent of the metaphase figures show a value at exactly 46 (diploid) (50 cells counted per culture with aberrations less than 2 per cent). Tables 2 and 3 illustrate the effects of lysates from cells containing

TABLE 1

Diploid human peripheral blood leukocyte cultures at the time of establishment*

No.	Original culture	Established	Interval (days)	Sex	Chrom/cell (100 cells) 44	45	46	47	Polyploid
1	10.20.67	12.25.67	66	M		3%	94%		3%
2	11.01.67	12.26.67	55	M	3	3	92		2
3	11.03.67	12.21.67	48	M		5	94		1
4	10.14.67	12.26.67	73	M	1	4	91		2 (endo-reduplic)
5	11.10.67	1.03.68	55	M		3	95		2
6	7.28.67	1.03.68	159	M		2	97		1
7	10.26.67	1.09.68	75	M		1	99		0
8	10.30.67	1.09.68	71	F	1	2	94		3
9	11.17.67	1.02.68	49	M	3	2	93	1	1
10	11.04.67	1.09.68	66	M	1		98		1
11	11.15.67	1.09.68	55	M	1	1	95		1
Averages			70·18						1·73%

* See: Proposed usage of animal tissue culture terms: Committee on Terminology, 1966 (in References)

EBV, the herpes-like virus found in approximately 1/2 of both normal and leukaemic buffy coats from peripheral blood. There were no distinct chromosomal lesions, however, increased polyploidy occurred. The type C chromosome abnormality described for virus infection was not encountered.

In other studies with haematopoietic cells from several sources cultures could be grown which demonstrated what appeared to be a helper activity for *in vivo* expression of a mouse leukaemia virus (Friend virus). Although cultures from human peripheral blood, bone marrow, lymph nodes, and

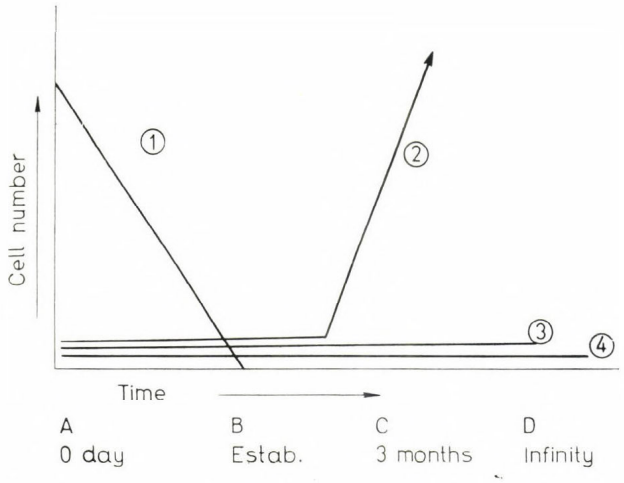


Fig. 1

TABLE 2

Diploid human peripheral blood leukocyte cultures at the time of establishment treated with cell lysates from cell containing HLV

Culture No.	Sex	Polyploid %
1	F	3
2	F	4
3	F	3
4	F	5-8
5	M	4–5
6	M	0–15
7	M	1–2
8	M	1–3
9	F	1–2
10	M	12
11	M	1–2
12	M	0–5
13	F	0–1
14	F	0–1
15	M	0–1
16	M	1–2
17	F	1–2
18	F	1–2
19	F	1–2
20	M	0–3
21	M	3–8
22	M	1–4
23	M	4–8
24*	F	0–1

* Stem line = 46 (60 per cent) and 47 (40 per cent) with minute chromosome abnormality present in three-day PHA-treated cultures, therefore, not the result of lysate treatment.

TABLE 3

Peripheral blood leukocyte cultures at time of establishment, treated with cell lysates in humans

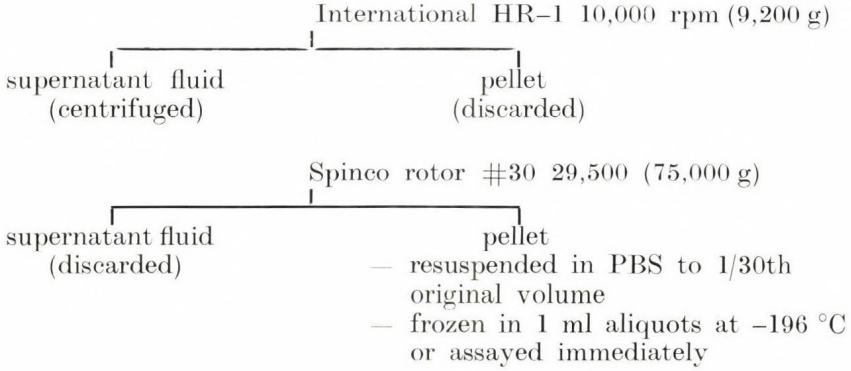
Culture No.	Sex	Stem line
1	F	diploid*
2	F	diploid*
3	M	diploid*
4	M	diploid*
5	M	diploid*
6	M	diploid*
7	M	diploid*
8	M	diploid*
9	F	diploid*
10	F	diploid*
11	M	diploid*
12	F	diploid*
13	M	diploid*
14	M	diploid*
15	F	diploid*
16	M	diploid*

* Indicates more than 75 per cent of the metaphase figures show a value of exactly 46 (diploid). Fifty well spread metaphase figures per culture examined. Less than 2 per cent of the metaphase figures had aberrations.

spleen, showed numerous transformed blast type cells, the activity has been limited to spleens from donors with chronic myelogenous leukaemia, Hodgkin's disease, and giant follicular cell sarcoma. At the present time material from 61 human donors has been investigated, including normal embryos (negative) and normal adult spleen (negative). Spleens from 4 of 6 individuals with chronic myelogenous leukaemia have shown evidence of helper activity which is increased during a 48-hour cultivation period. The following protocol shows the system used in this study. These studies were done in collaboration with Dr. R. Steeves and Dr. E. Mirand.

PROTOCOL FOR PREPARATION OF HUMAN SPECIMENS

Spleen (20 g)
- minced with scissors in Eagle medium and 10 per cent FCS;
- first wash discarded (mainly RBC's), but succeeding washes with each mince placed in separate bottles and cultures;
- final mince of tissue fragments cultured in "Maitland" set-up;
- incubated at 37 °C for 48 h and fresh medium added p.r.n.;
- pooled culture fluids centrifuged 10 min.



VARIABLES TO BE TESTED

(1) Donor tissue—from normal vs. leukaemic patients; clinical status and therapy.

(2) Cell separation—mincing vs. trypsinization; WBC vs. fibroblastic cultures.

(3) Media—Eagles', 1630, 199; concentration of FCS or HS.

(4) Culture conditions—partial pressure of CO_2; with or without PHA.

(5) Centrifugation procedure—biological activity of discarded pellet or supernatant fluid.

(6) Biological activity—helper activity for SFFV vs. direct spleen focus-forming activity.

(7) Indicator virus stock – SFFV(GLV) pseudotype vs. human pseudotype of SFFV.

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ULTRASTRUCTURAL FEATURES OF GONADOTROPIC PITUITARY CELLS IN CULTURE

by

A. TIXIER-VIDAL
LABORATOIRE DE BIOLOGIE MOLÉCULAIRE, COLLÈGE DE FRANCE, PARIS, FRANCE

A study of ultrastructural features of gonadotropic pituitary cells in culture must be placed into a functional context: our present concept of regulation of gonadotropic function.

At the pituitary level, three hormones participate in the regulation of gonadal activity. Two glycoprotidic hormones, follicle stimulating hormone (FSH) and luteinizing hormone (LH), control, in the female, the growth, secretion and rupture of ovarian follicles, and, in the male, the spermatogenesis and the maturation and secretion of interstitial cells of the testis. One protidic hormone, prolactin, discovered by its effect on the mammary gland, acts also at several phases of sexual activity. In the pituitary tissue each one of these three hormones is synthesized by a distinct cell type, characterized by its staining affinities, cytochemical properties and ultrastructural features. Among the latter, the mean size of secretory granules, the structure of the Golgi zone and of the rough endoplasmic reticulum (RER) allow to distinguish between the cell types and to estimate the level of their secretory activity.

The secretion of each of these three hormones is under the control of neuro-hormones which are produced in the hypothalamus and then carried to the anterior pituitary by the portal vessels. Three different factors have been isolated from bovine, ovine or porcine hypothalamus and identified by their effects on the release of each one of the three gonadotropic hormones: the FSH releasing factor (FRF) stimulates the FSH release, the LH releasing factor (LRF) stimulates the LH release, the prolactin inhibiting factor (PIF) inhibits the prolactin release. Nevertheless in birds, prolactin secretion is also dependent on a stimulatory hypothalamic factor (PRF).

Lastly, in addition to hypothalamic factors, sexual steroids act also on gonadotropic pituitary cells and, therefore, on the secretion of gonadotropic hormones.

Such complex regulations need to be analysed in *in vitro* experiments. In fact, the first known releasing factor 'corticotropin releasing factor' was discovered by Guillemin and Rosenberg (1955) in pituitary culture experiments. *In vitro* methods have been and are currently used in this research field. However, slice incubation techniques are more often employed than regular cultures, such as organ culture, tissue culture, and cell cultures. In this paper, we will only deal with these last named methods.

In spite of their great interest, only few data are available on the ultrastructure of cultured pituitary cells. Two important contributions to this field were published almost simultaneously. Petrovic found (1959, 1961,

1963) in guinea pig and rat pituitaries that in culture each one of the different cell types keeps its specific ultrastructural features in spite of the rapid disappearance of secretory granules. Nevertheless, except in 'epsilon' cells, ergastoplasm and Golgi zone are reduced, indicating a low, basal activity. According to this author, functional differentiation of all pituitary cell types is maintained in culture, but their storage function is abolished. Pasteels (1963) ascertained, in rat and human pituitary cultures, ergastoplasm extension of a cell type that he identified as prolactin cells, by showing an increasing rate of prolactin production in the culture medium. As to the other cell types, including gonadotropic cells, Pasteels described their progressive atrophy and disappearance in culture. These two authors drew, therefore, opposite conclusions concerning pituitary cells submitted to treatment with stimulatory hypothalamic factor.

In this paper, we will describe ultrastructural features of pituitary cells in culture in three different biological models. Moreover, in order to attempt a dynamic interpretation of the static ultrastructural data, we will compare them with results of hormone assays and with results of tracer incorporation into proteins.

ULTRASTRUCTURAL FEATURES OF DUCK PROLACTIN CELLS IN ORGAN CULTURE ANALYSIS OF THE SECRETORY CYCLE

Physiological data

In the male duck, there is no specific and measurable receptor for pituitary prolactin. In order to study the neuro-endocrine regulation of this hormone, it was necessary to use pituitary cultures. They were performed on synthetic medium supplemented with 10 per cent duck serum and 5 per cent chick embryo extract, either by the hanging drop method (Tixier-Vidal and Gourdji, 1965; Tixier-Vidal and Picart, 1967) or on stainless grids in Falcon organ culture dishes (Tixier-Vidal and Gourdji, 1970). As bioassay for prolactin, the micro-method on pigeon crop-sac was used.

In the first step we have shown that prolactin is produced at a constant rate during the first and second weeks of culturing, and that the addition of a hypothalamic extract to the medium increases prolactin production threefold (Gourdji and Tixier-Vidal, 1966).

In order to estimate the secretion rate of prolactin and its renewal time in culture, we have compared prolactin tissue content before cultivation, after one week and after two weeks of cultivation on one hand, to the prolactin level in culture media after the first and the second week on the other (Tixier-Vidal and Gourdji, 1970). The results (Fig. 1) allow the following conclusions:

(1) Prolactin is present in the culture medium at a constant level during the first and second weeks of cultivation.

(2) The amount of prolactin liberated into the medium each week is clearly greater than the initial content of the gland, in fact, three-, or sixfold.

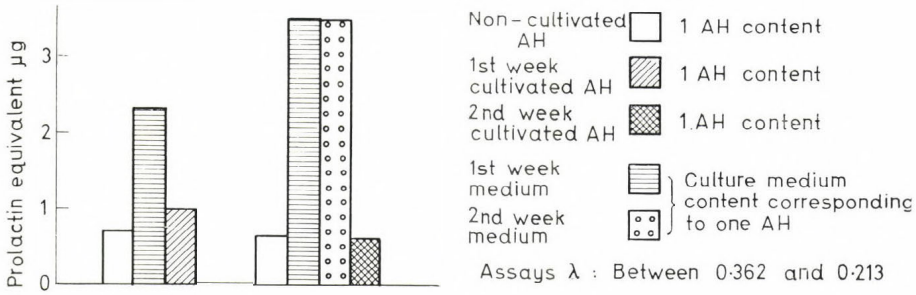

Fig. 1. Prolactin secretion by duck pituitaries maintained in organ culture for one and two weeks. AH = adenohypophysis

(3) The amount of prolactin present in the tissue after one week and after two weeks of cultivation is constant and equal to the initial amount in the uncultured gland.

It can, therefore, be concluded that *de novo* synthesis of prolactin occurs in culture. The renewal time of prolactin thus synthesized can be estimated at 48 h or 24 h depending upon the experiments. This time would, however, be shorter if partial destruction of prolactin occurred either in the media after excretion, or in the tissue before excretion.

Ultrastructural data

Tissue cultured for 1 or 2 weeks shows a simplified structure. Among the 7 cell types previously distinguished *in situ* (Tixier-Vidal, 1965), only two retain their specific characters in culture. One of them (type I) is more frequent and is identified by its polymorphic secretory granules, the size and number of which greatly differ from one cell to another. The well-developed ergastoplasm, Golgi zone and the presence of granule exocytose pictures, allow to assume that such cells are able *in vitro* as *in vivo* to synthesize and to release a hormone (Fig. 2). The second cell type is less frequent. These cells are smaller and contain spherical granules (mean dia. : 200 to 250 mμ). The ergastoplasmic cisternae are always flat and Golgi saccules are arranged in a ring. These cells look like gamma or kappa cells but they are far less developed than in normal pituitary tissue.

The predominance and the level of activity of type I cells led us to consider them prolactin cells, and we used them as a model for a finer analysis of the secretory cycle with a labelled precursor.

Protein cycle in duck pituitary cultures

In order to study the protein cycle in our cultures, we examined the incorporation of tritiated L-leucine into tissues and secretory proteins. After a short pulse (5 min or 10 min) we followed by high resolution autoradiography the migration of newly synthesized proteins either in the cell structures (Tixier-Vidal and Picart, 1967) or in the tissue and in the culture media, after

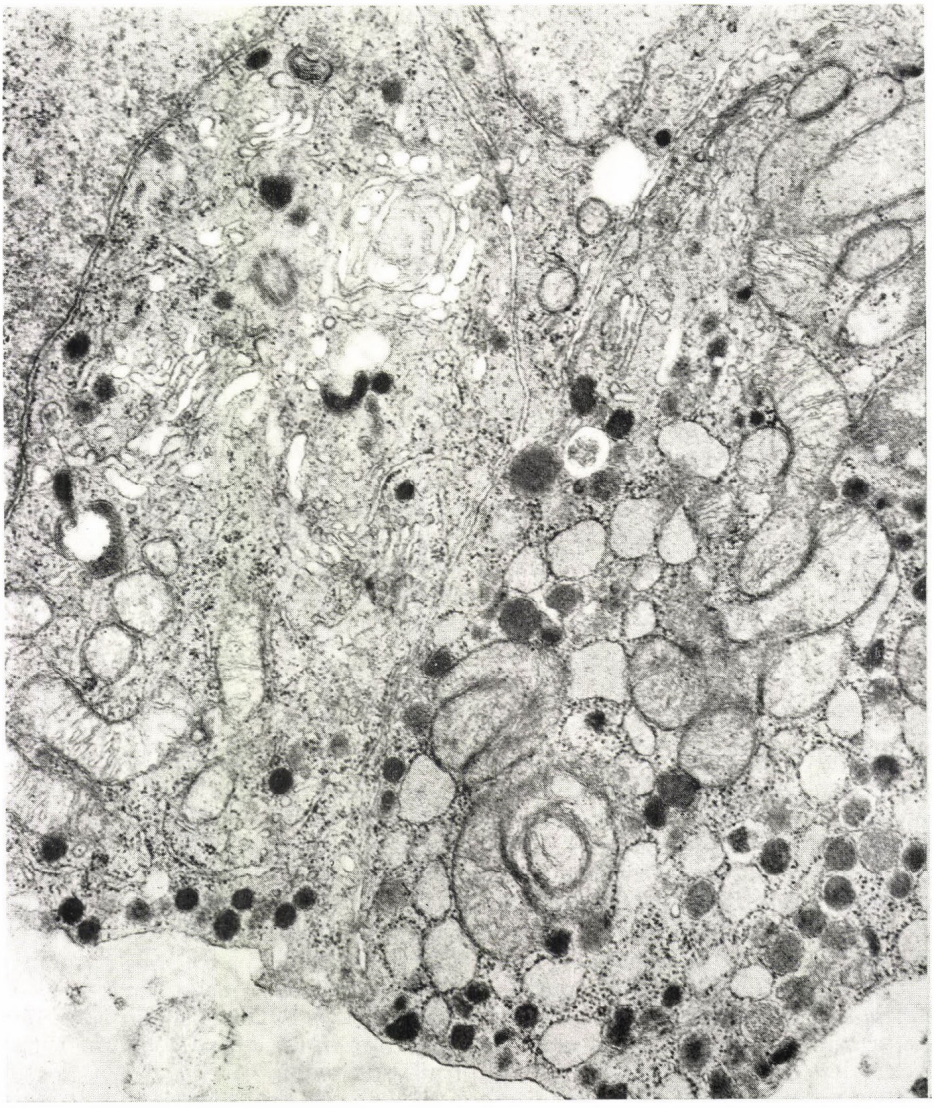

Fig. 2. Prolactin cells in duck pituitaries cultured for two weeks. Notice the development of the Golgi zone and the two different forms of the ergastoplasmic cisternae. Material fixed with glutaraldehyde and osmic acid. Section contrasted by uranyl acetate and lead citrate. ×26,000

TCA precipitation and counting of the protein radioactivity (Tixier-Vidal and Gourdji, 1970). With high resolution autoradiography, we found that the newly synthesized intracellular proteins follow the classic pathways already shown for other glandular cells. After their synthesis in the ergastoplasm they are concentrated in the Golgi zone within 30 min (Fig. 3). They then migrate out of this area, the emptying of which is accomplished

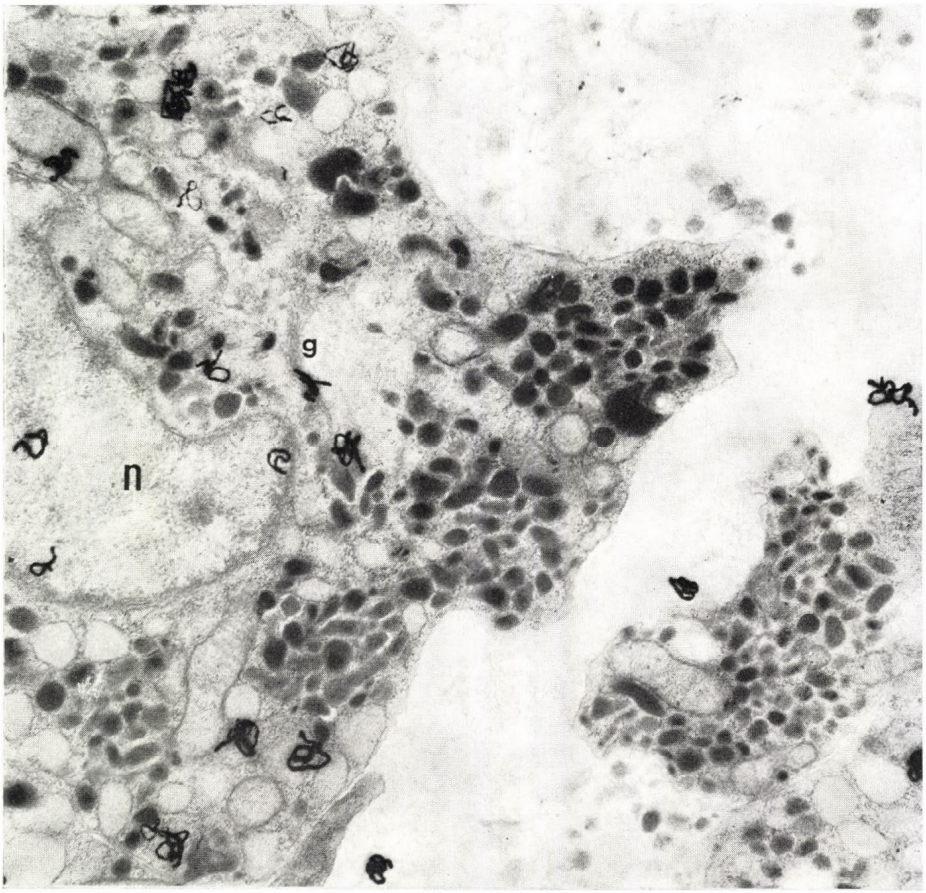

Fig. 3. Prolactin cells in duck pituitaries cultured for 12 days. Tissues are fixed 20 min after the beginning of the chase, following a 15 min pulse in the presence of tritiated L-leucine. Tissues are prepared for autoradiography. The radioactivity is seen in the nucleus, Golgi zone and cytoplasm or ergastoplasm. The secretory granules are not labelled. $\times 17{,}000$

in about 4 h (Fig. 4). These proteins become distributed in equal amounts between the protein granules on the one hand, and the cytoplasm (sedentary proteins) on the other. Several peculiarities seem to distinguish pituitary cells from others, at least in culture: (*i*) the emptying of the Golgi zone is slow, (*ii*) the percentage of labelled granules is low (max. 3 per cent) and then decreases slowly (1 per cent in 40 h), (*iii*) a large portion of newly synthesized proteins (40–50 per cent) remains localized in the cytoplasm outside the secretory granules.

As far as the whole culture is concerned, it can be concluded from a quantitative study of the events during the chase of the radioactivity of proteins in the tissues and in the culture media, that:

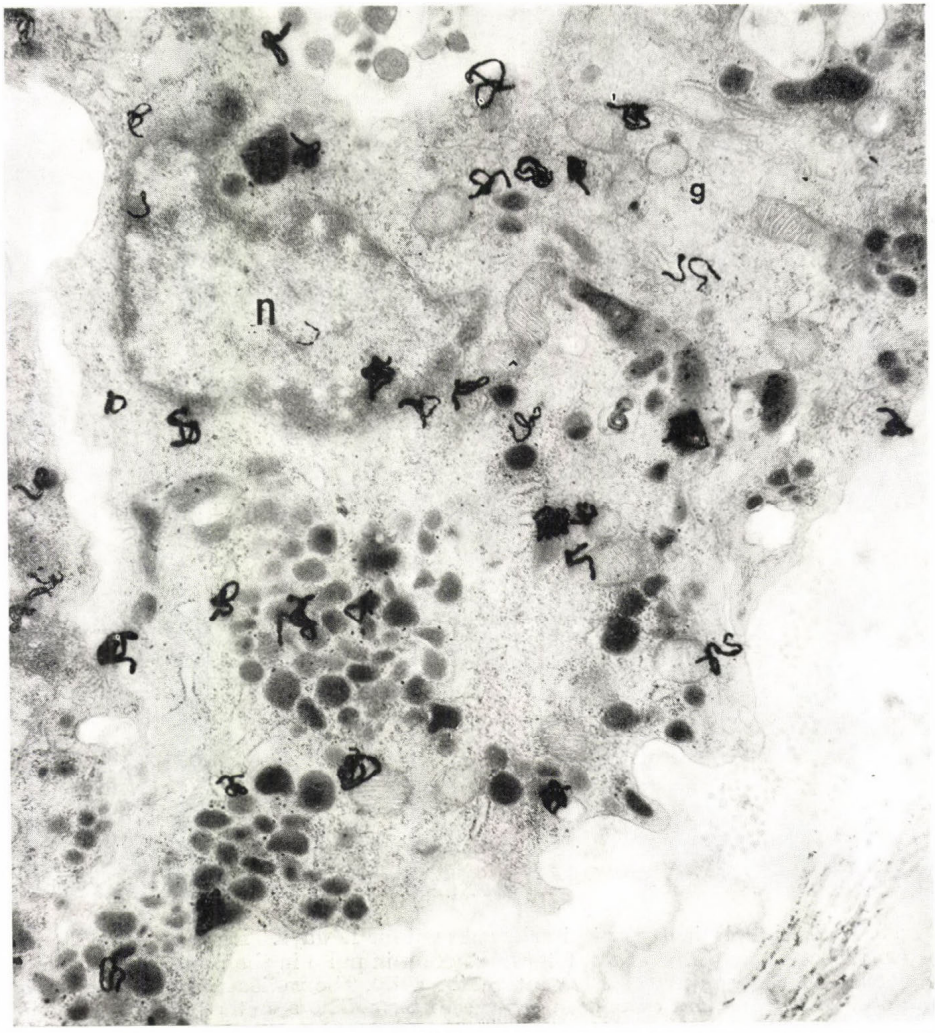

Fig. 4. Same material as in Fig. 3, but here fixation was made after 1 h of chase. The Golgi zone contains only few silver grains, but in contrast to Fig. 3 several secretory granules are labelled. ×18,000

(1) The tritiated leucine molecules are incorporated very rapidly into tissue proteins.

(2) The radioactivity of these proteins is maximum at the beginning of the chase. This maximum is followed by a two-phase decrease, a rapid first phase of 30 min or 1 h, and a slower second phase.

(3) The radioactivity of proteins in the culture medium begins to increase after 30 min or 1 h of chase.

It, therefore, appears that, in our cultures, neosynthesis of proteins is followed by migration and excretion into the medium. The delay between

the synthesis in the tissue and the appearance of radioactive proteins in the medium seems to correspond to their intracellular migration from ergastoplasm to secretory granules. Therefore, synthesis and release are two distinct phenomena.

In conclusion, the use of three different approaches: bioassay, high resolution autoradiography and the kinetics of tracer incorporation into total proteins, allow to characterize the biosynthetic and secretory capacity of duck prolactin cells in organ culture.

ULTRASTRUCTURE OF LH CELLS IN RAT PITUITARIES CULTURED FOR TWO WEEKS IN THE PRESENCE AND ABSENCE OF LRF

Physiological data

Our kinetic data on LH secretion by rat pituitaries maintained in organ culture (Tixier-Vidal et al., 1970) show without any doubt that this model is quite different from that of duck prolactin cells.

Male rat pituitaries were cultured in Falcon organ culture dishes (one pituitary per dish) using 199 synthetic medium supplemented with 10 per cent calf serum, 5 per cent foetal calf serum and 5 per cent chick embryo extract. Purified LRF was added to this medium in two different doses: either 0·5 μg or 2 μg/ml, and renewed every 2 or 3 days. LH was measured by radio-immunoassay (Kerdelhué et al., 1970). The sensitivity of this test allows to obtain individual data for each cultured pituitary, both for the tissues and for the media, as a function of the time of culturing. In the present paper, we only deal with 3 series of 12 pituitaries cultured for 12 or 14 days. In control cultures, LH level in the media greatly decreased during the first week and thereafter had a tendency to remain constant or to slightly increase. The tissue content at the end of the culture period was much lower than the initial content. Moreover, the total LH harvested in culture (tissue + medium) is also lower than the initial content. This is evident in series C. 69. In series C. 65 and C. 66 the total LH production was higher, but did not differ considerably from the mean LH content of male rat pituitary (between 5,000 to 10,000 ng/AP).

When pituitaries were cultured in the presence of LRF from the third day to the end (12th or 14th day), we noticed a marked increase of the release of LH in the medium. At the same time, the LH tissue content was constant or slightly increased. Nevertheless, even under these conditions, the total LH production (tissue + medium) did not clearly exceed the pituitary initial content.

These data indicate that in the culture some of the LH are destroyed, the extent of which depends more or less on the series. Nevertheless, we notice, from C. 69, that a low LH production does not prevent a high reactivity to LRF; this suggests a healthy state of the cultures.

It, thus, appears that in this model the results of hormone assay are not sufficient to establish whether or not there occurs a synthesis of LH in the absence or in the presence of LRF.

Ultrastructural features

(*a*) In *control cultures* we found hyperactive prolactin cells as previously described by Petrovic and Pasteels. These cells are recognized by their well-developed flat ergastoplasmic cisternae, arranged in parallel rows or sometimes in *Nebenkerne*. Secretory granules are generally sparse, but more numerous in some cells. They are voluminous (mean diameter : 250 to 300 mμ), rounded or ovoid (Fig. 5).

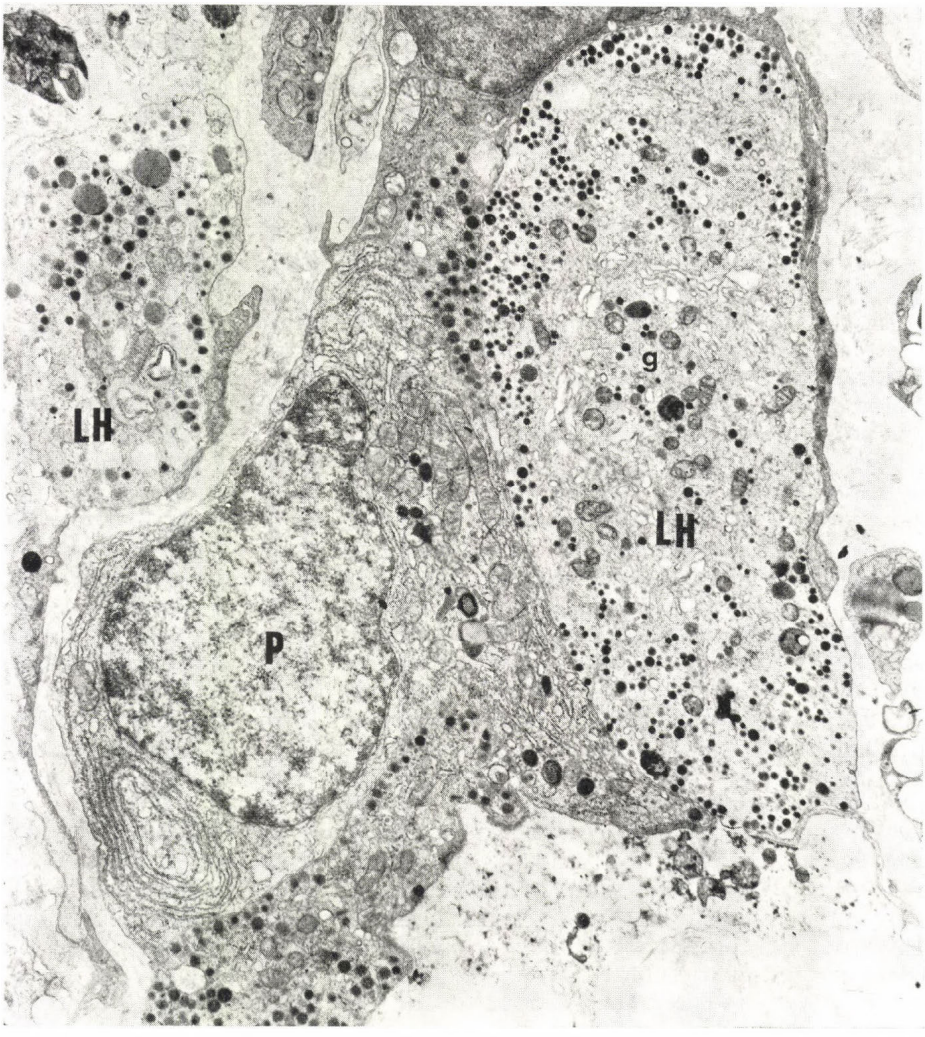

Fig. 5. Rat pituitaries maintained in culture on a control medium for 14 days. P, a prolactin cell with numerous flat ergastoplasmic cisternae arranged in parallel rows. LH, cells containing two granule populations differing in their respective mean diameters; the rough reticulum is not developed, the Golgi zone is not atrophied. Material is fixed with glutaraldehyde, osmic acid and uranyl acetate. Sections are contrasted with lead citrate. × 9,000

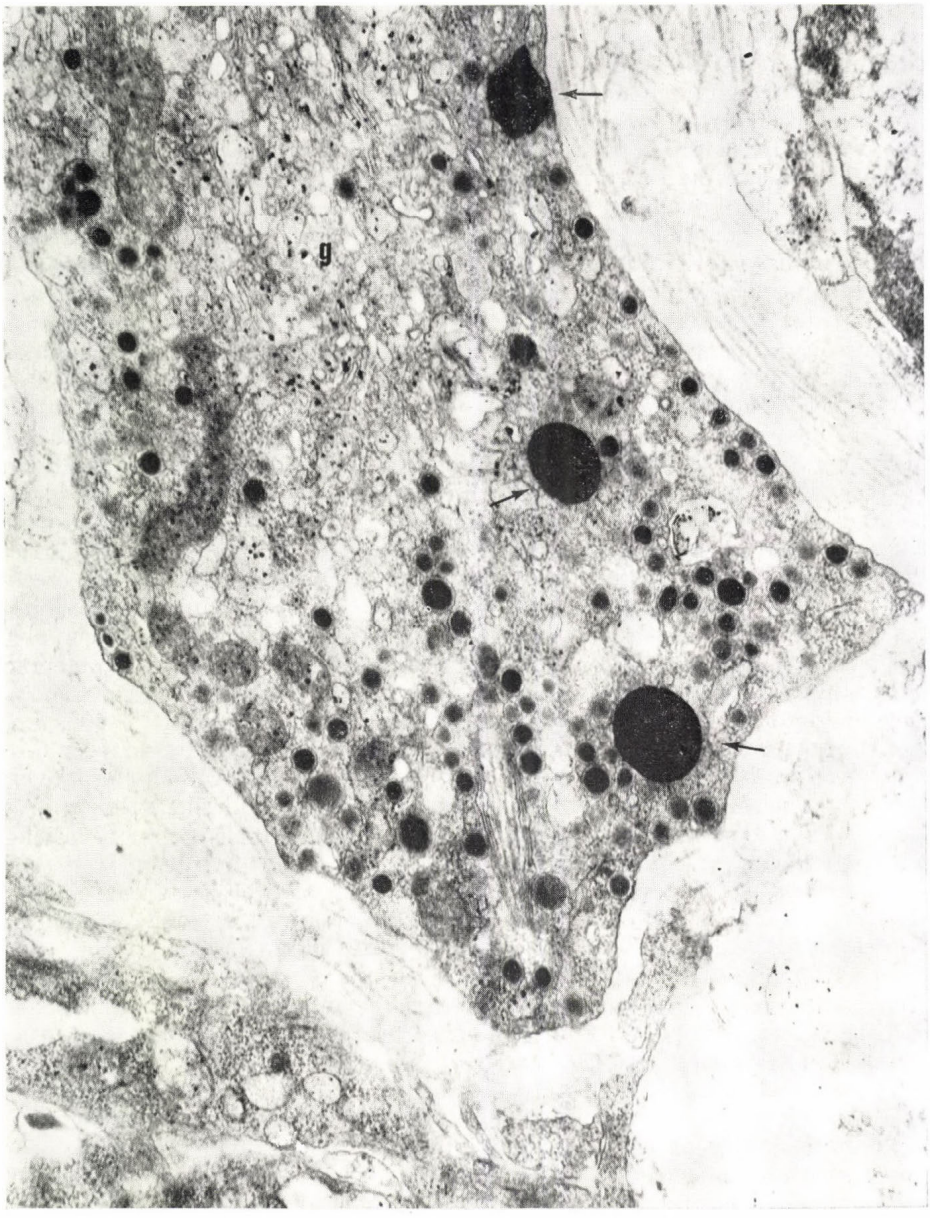

Fig. 6. Same material as in Fig. 5. Tissues prepared for detection of acid phosphatase activity by the Gomori method. The reaction product is abundant in large granules (arrows). They are scattered in the Golgi zone and in some mitochondria. × 26,000

Between prolactin cells, other cell types are scattered. Among them we indentified LH cells on the basis of their features previously defined in rat pituitary *in situ* (Farquhar and Rinehart, 1954; Herlant, 1964). Two characters draw our attention: the linear or bent ergastoplasmic cisternae, and the two populations of granules differing in size. In non-cultured rat pituitaries LH cells contain two granule populations differing by their respective mean diameter : 150 to 200 mμ and 400 to 550 mμ. In tissue maintained two weeks in culture, LH cells are small, rounded or ovoid (Fig. 5). They generally have numerous small granules mixed with scattered large granules (until 700 mμ dia.). In sections prepared for the localization of acid phosphatase these large granules are rich in reaction product and might thus considered lysosomes (Fig. 6). In such LH cells, the ergastoplasmic cisternae are scarce and the Golgi zone is small though not atrophied (Fig. 5).

On the whole, LH cells, in tissue cultured without LRF, retain ultrastructural features suggesting a possible persistence of some synthesis. Moreover, the presence of numerous lysosomes could indicate an intracellular destruction of LH the release of which is low. In addition to this intracellular destruction, other processes could explain the loss of LH during the entire period of culturing, such as (*i*) necrosis of some LH cells particularly during the first days of culture, when LH release is greatly decreasing and (*ii*) a possible proteolysis of LH after its release into the medium.

(b) *In culture maintained on LRF-containing medium*, after two weeks, the appearance of LH cells is greatly modified. They take on an amaeboid shape and they penetrate among the always active prolactin cells (Fig. 7). The diameter of the small granulations decreases (to less than 100 mμ) and they are gathered at the cell periphery. The large granulations (lysosomes) appear less numerous and less rich in enzyme reaction product. Moreover, the flat ergastoplasmic cisternae are well developed and the Golgi zone is largely expanded with several stacks of saccules, numerous microvesicles and segregating secretory granules (Fig. 8). Such pictures suggested that the increased LH release observed in the presence of LRF is, at least partly, the result of a high stimulation of the cytoplasmic organelles such as ergastoplasm and Golgi zone involved in synthetic processes.

In summary, two tentative conclusions can be drawn from the ultrastructural study of LH cells in cultured rat pituitaries : (1) in the absence of LRF they are capable to maintain a certain level of hormonal synthesis, (2) in the presence of LRF this synthesis is highly stimulated.

These conclusions ought to be ascertained by other methods. With this object in view, we have initiated (in collaboration with Dr. Jutisz's group) the kinetic study of incorporation into LH of tritiated proline by rat pituitaries previously cultured for 7 days. The tritiated LH is isolated by immunological methods (Kerdelhué et al., 1971). Our first results clearly show that neosynthesis occurs in culture and in the absence of LRF.

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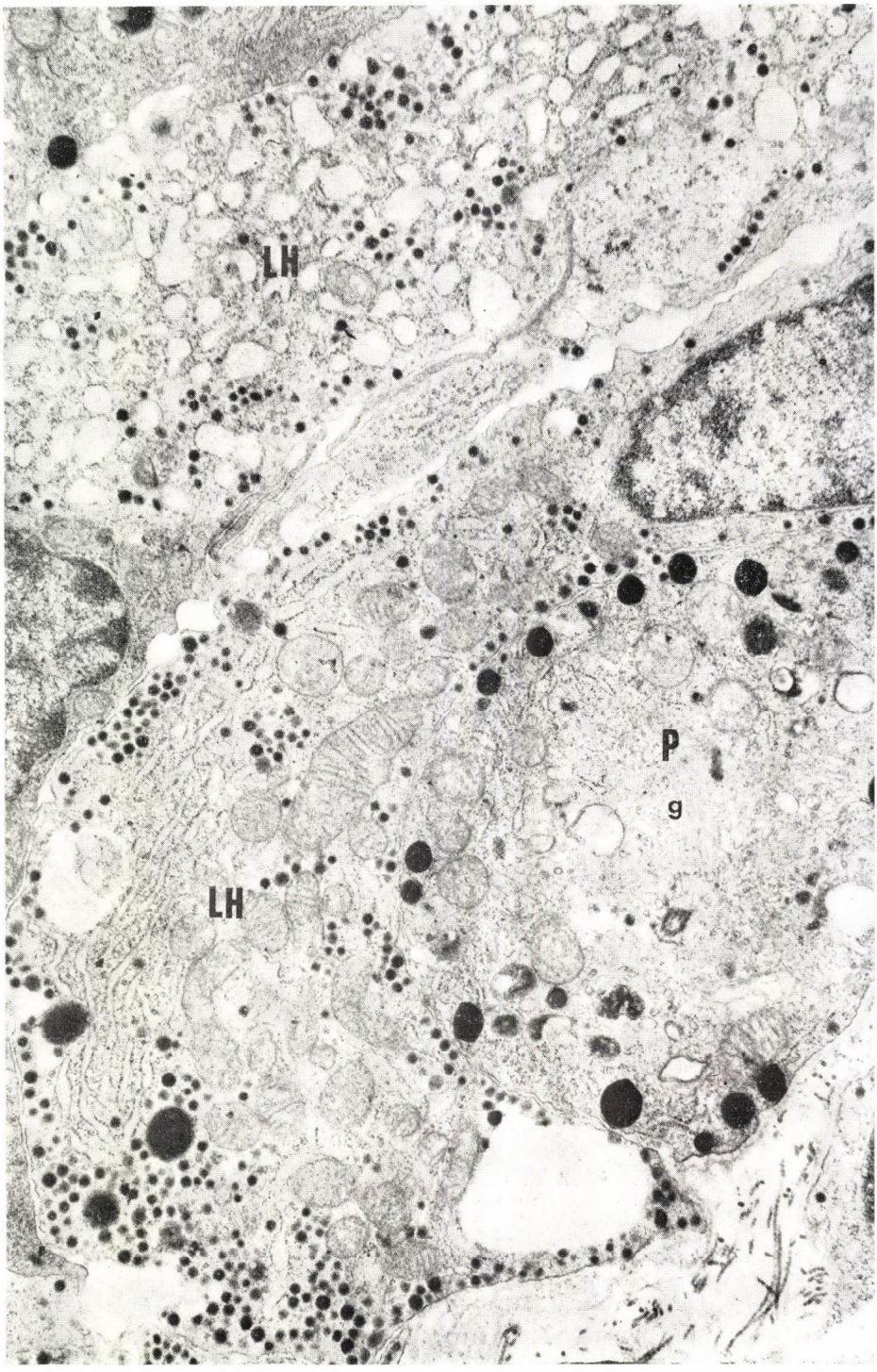
Fig. 7. Rat pituitaries maintained in culture from day 2 to day 14 in the presence of LRF (2γ per ml renewed each 2 or 3 days). P, prolactin cell with well-developed Golgi zone (g). LH cells are much more developed than in Fig. 5. Note the appearance of the ergastoplasmic cisternae either irregular or in parallel rows, and the decreasing diameter of the small granules. Same techniques as in Fig. 5. × 14,000


LH
P
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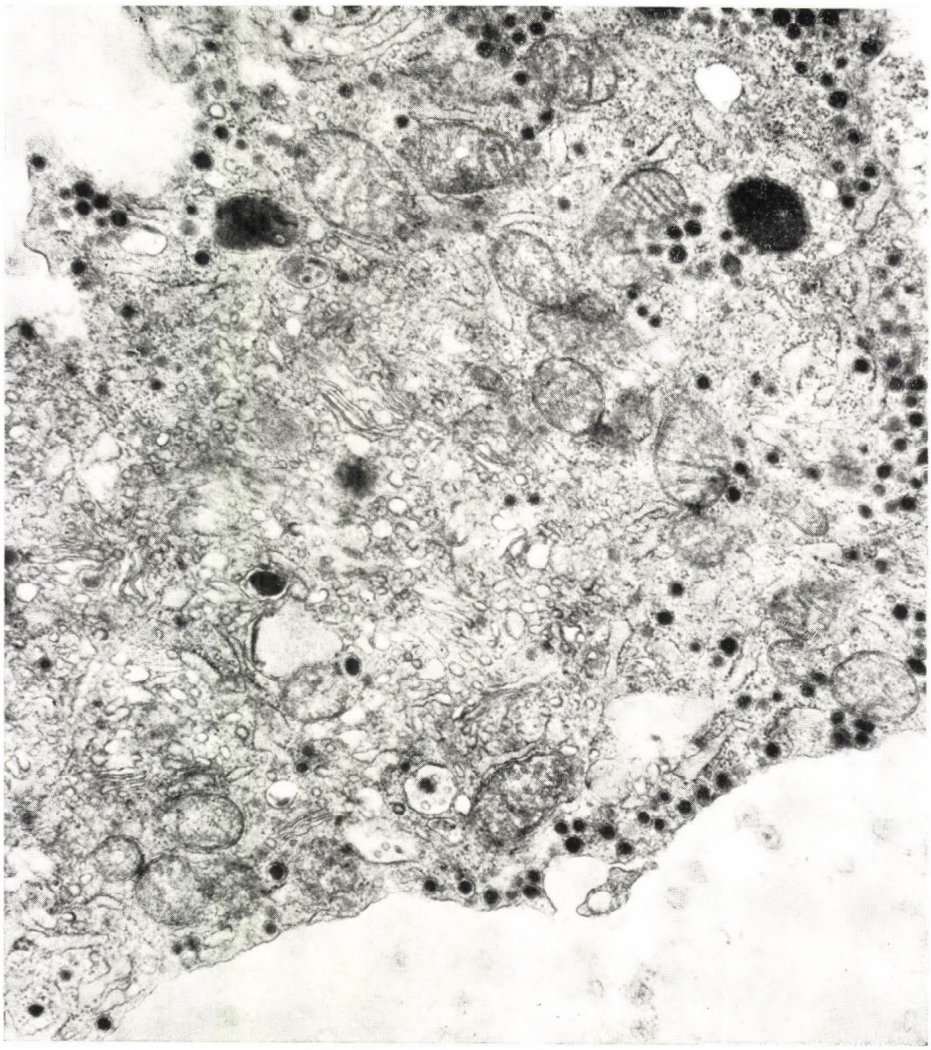

Fig. 8. Same material as in Fig. 7. Tissue prepared for detection of acid phosphatase activity. Detail of the Golgi zone of a stimulated LH cell. Compare with Fig. 6. The Golgi zone is largely expanded with numerous saccules and vesicles lacking reaction product. The large granules are almost devoid of reaction product. The small secretory granules are lined along the cell membrane. $\times 26{,}000$

ULTRASTRUCTURAL FEATURES OF RAT PITUITARY CELLS OF THE GH3 STRAIN

It seemed interesting to compare the two previous models with some recent results obtained in my laboratory by Gourdji (1971) concerning pituitary cells from the GH3 strain maintained in cell culture for several years. The GH3 strain was established by Tashyian et al.(1970) from pituitary

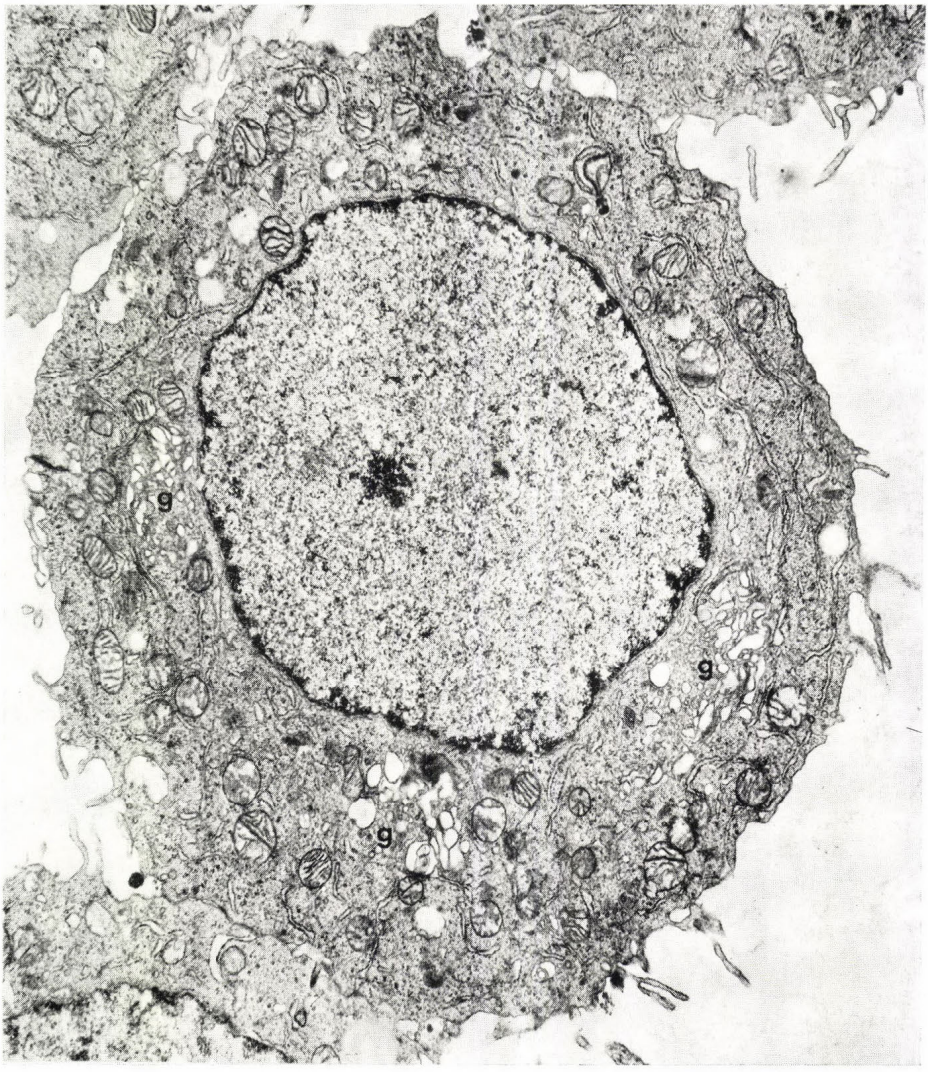

Fig. 9. Rat pituitary cells of the GH3 strain. Cells growing in suspension. The secretory granules are extremely scarce. The Golgi zone is composed of 3 subunits. The flat ergastoplasmic cisternae are scattered. Fixation: glutaraldehyde, osmic acid, uranyl acetate. Sections are contrasted with lead citrate. $\times 10{,}000$

tumours induced by oestrogen in Wistar–Furth female rats. According to Tashyian et al. (1970) the GH3 strain produces prolactin and growth hormone simultaneously. Dr. Sato sent us a sample of this strain and we could maintain it in our laboratory for some months. We have confirmed by pigeon crop-sac bioassay the high level of prolactin production in the medium.

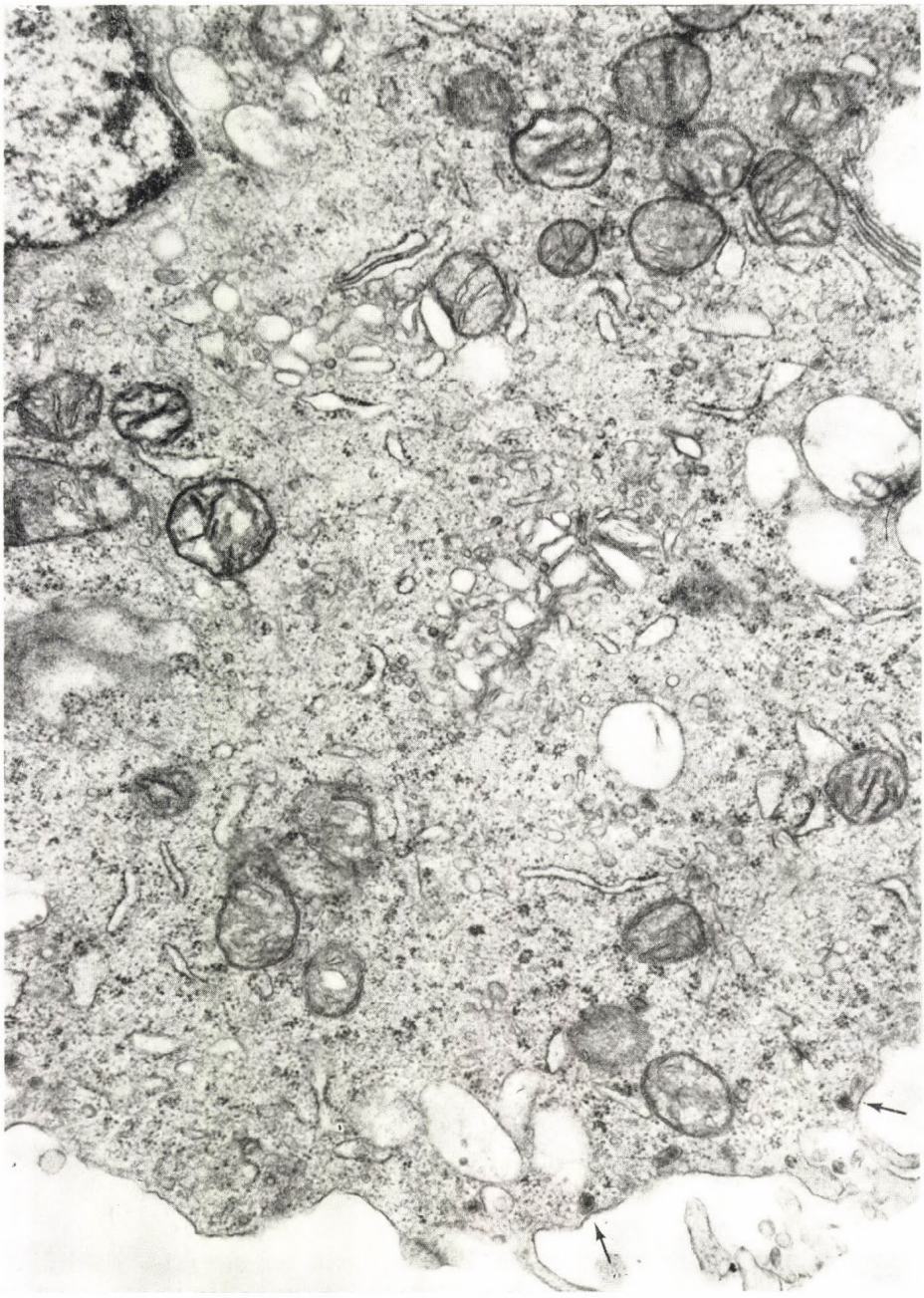

Fig. 10. Same material and same techniques as in Fig. 9. A detail showing the smooth reticulum in the Golgi zone and the numerous polysomes in the cytoplasm. Here again the secretory granules are very rare, and they are localized along the cell membrane (arrows). $\times 44{,}000$

This model differs from the two models described previously in two important points : (1) the high rate of cell division (generation time : 20 to 30 h) and (2) the brief renewal time of prolactin content : 1 h according to Tashyian et al. (1970). This value is far less than that obtained from *in vivo* data : 91 h in male rat, 21 h in oestrogenized female rat, 10 h in grafted rat pituitaries (Nicoll and Swearingen, 1970).

Under the electron microscope, these cells have been seen to have very few small granules, either dispersed or lined along the cell membrane (Fig. 9). In the cytoplasm, polysomes and ribosomes are extraordinarily abundant (Fig. 10) but the flat ergastoplasmic cisternae are less numerous than in prolactin cells from pituitary organ cultures. The Golgi apparatus is divided into 2 or 3 subunits composed of saccules and microvesicles, but condensing secretory granules are unfrequent (Fig. 10).

The pictures are quite different from those obtained with active rat prolactin cells either *in vivo* or in organ culture. Here the ergastoplasmic cisternae are less developed, the Golgi zone is divided into several subunits, granule segregation in the Golgi zone and granule exocytosis are scarce. All these features are in good correlation with a low hormone content. Nevertheless, the shortness of the renewal time of the prolactin cell content indicates intensive synthesis as well as release of the specific protein. This contrasts with the ultrastructural organization of these cells which almost look like 'embryonic' cells. It seems that in this case there is an important acceleration of the intracellular migration of the secretory protein. Some stages of this migration such as concentration in the Golgi zone and especially storage of secretory granules, might be skipped. The determinism of such a transformation of secretory process is unknown. One can put forward the loss of cellular interaction in cell culture or perhaps a previous malignant transformation which facilitates the establishment of cell strains. Further work is needed in order to answer these questions.

CONCLUSIONS

The final aim of studies with cultured cells is the better understanding of *in vivo* intracellular regulatory mechanisms. From this point of view, confrontation of the ultrastructural features of three different pituitary cells cultured *in vitro* definitely shows that it would be premature at present to propose a single model for the regulation of the secretory activity of the pituitary cells.

The ultrastructural criterion appears to vary in close correlation with the renewal time of the cells' hormone content. We do not know, however, which one of these two phenomena determines the other. Furthermore, these phenomena are, in turn, determined by the chemical nature of the specific hormone or by the type of control regulating *in vivo* the secretion of this hormone. Further research is still necessary in this field, especially as far as the mechanism of pituitary cell differentiation is concerned.

*

Acknowledgements. We are indebted to Dr. Philippe Benda for showing us the methods of cell culture and helping us to maintain the GH3 strain. We thank also for their excellent technical assistance, Mlle M. F. Mormiche and M. C. Pennarun.

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COMPARISON OF THE EFFECTS OF ACTH ON THE NUMBER OF MITOCHONDRIAL PROFILES IN CORTICAL CELLS AND ON THE CONVERSION OF PROGESTERONE-4-^{14}C INTO CORTICOSTERONE AND 18-OH-DOC IN TISSUE CULTURES OF FOETAL RAT ADRENALS*

by

A. I. Kahri, A. Lyytikäinen, S. Pesonen and A. Saure
2nd Department of Pathology, Department of Basic Zoology,
University of Helsinki, Finland

Based on two different protein synthetic systems, nuclear and mitochondrial *in vitro* studies have shown that ACTH is an inducer in the ultrastructural differentiation of cortical cells (Kahri, 1966) and mitochondria (Kahri, 1968, 1970, 1971; Kahri et al. 1970). However, recent findings indicate that the site of ACTH stimulatory activity is in the nucleus (Kahri, 1971). Other stimulatory effects on mitochondrial differentiation result from chloramphenicol-sensitive mitochondrial protein synthesis (Kahri, 1970), which is also mediated by cycloheximide-sensitive cytoplasmic ribosomal protein synthesis (Kahri, 1971). These results suggest the existence of a specific cytoplasmic mitochondrial protein synthesis stimulating factor which is probably a protein and which may be a mediator of ACTH action to the mitochondria. Corticosterone is the final product of the highly differentiated mitochondria of rat adrenocortical cells (Sharma et al., 1962; Pèron et al., 1966; Guerra et al., 1966; Kimura and Suzuki, 1967; Kimura, 1968), because the enzyme 11β-hydroxylase is located in the mitochondria and more specifically in the subfraction derived from their inner membranes (Yago and Ichii, 1969). *In vitro* studies also indicate that mitochondrial protein synthesis is partially responsible for the production of structural protein in cristae (Kahri, 1970) and that the ACTH-induced structural changes in the mitochondria correlate very well with the ACTH-induced increase in enzyme activity of mitochondrial 11β- and 18-hydroxylases in the cultivated cortical cells of fetal rat adrenals (Kahri et al., 1970).

To this end we have studied whether this ACTH induced increase in the capacity of cortical cells to hydroxylate progesterone at the 11β- and 18-positions were due to the changes in the number of mitochondria. Mitochondrial profiles were counted in cortical cells by light microscopy and the metabolites of progesterone-4-^{14}C were simultaneously analysed from the corresponding medium.

Twenty-one-day-old foetal albino rats of the Spraque–Dawley strain were sacrificed by decapitation. Their adrenals were explanted as tissue cultures. The average amount of tissue per culture was about 7 to 10 adrenals. The culture method employed was found in earlier studies (Kahri, 1966) to be suitable for long-term cultivation of adrenals. The medium contained 50 per cent Melnick "A" (0·5 per cent lactalbumin hydrolysate in Hanks' Balanced Salt Solution), 25 per cent calf serum (heat inactivated at 56 °C for 30 min and Seitz-filtered) and 25 per cent Eagle's Minimum Essential

* Supported by grant from the Sigfried Juselius Foundation and Paulo Foundation.

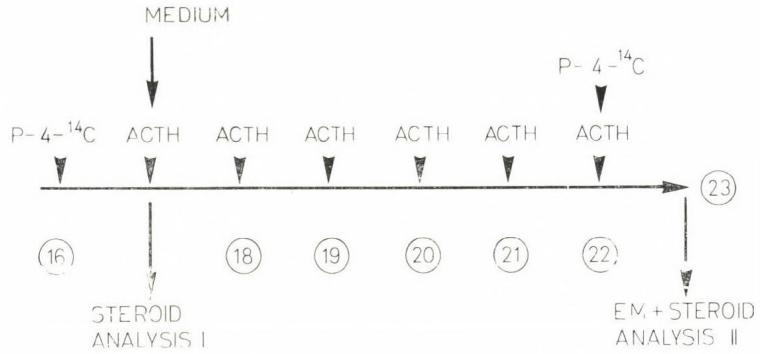
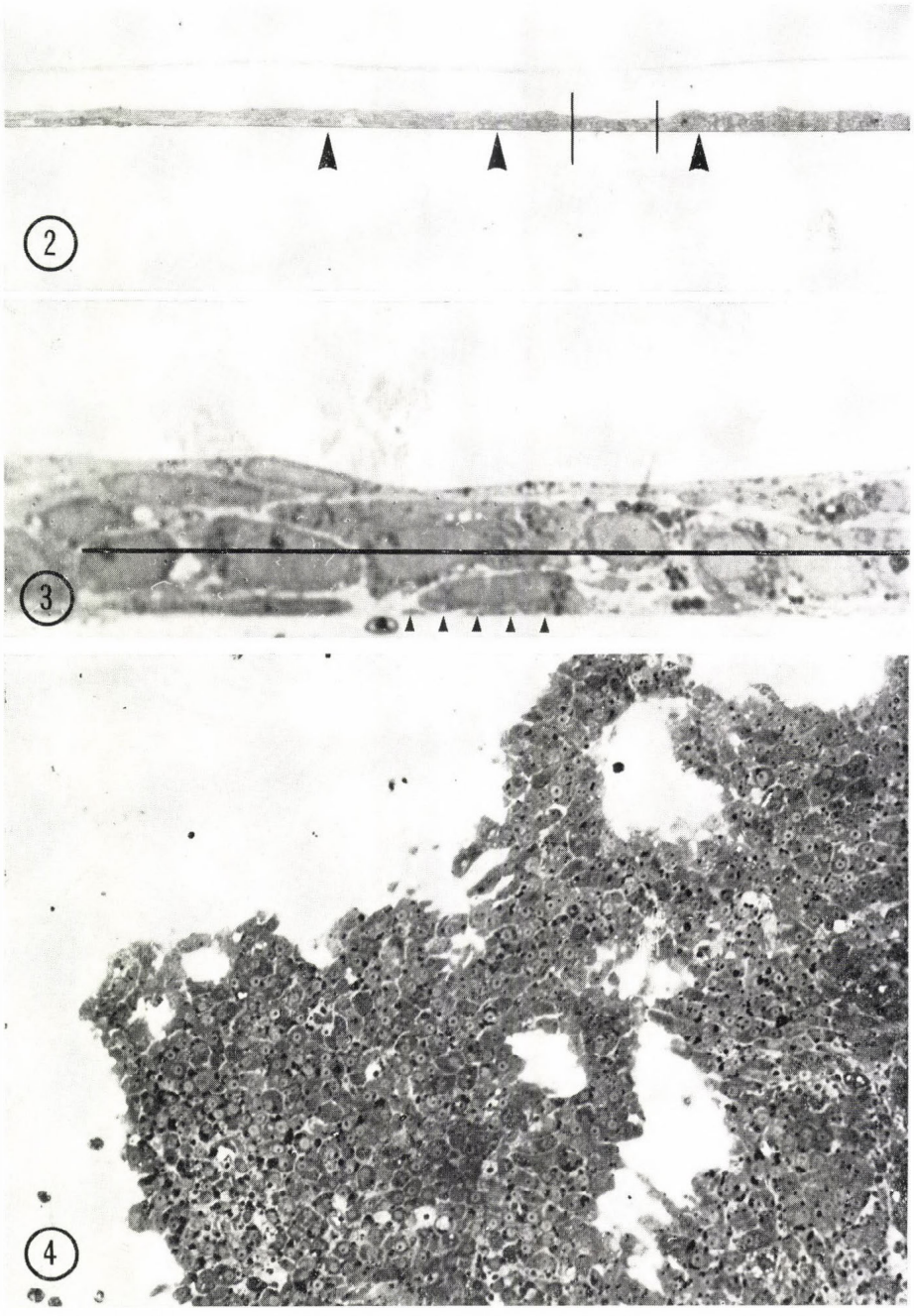


Fig. 1. Schematic representation of the design of tissue culture experiments. On the 16th day of cultivation 0·20 μCi of progesterone-4-^{14}C was added to the culture medium. On the 17th day, i.e. after 24 h, the medium was replenished and old medium analysed for progesterone metabolites. Every day from the 17th day on, up to and including the 22nd day of cultivation ACTH was added to the medium of some cultures. On the 22nd day of cultivation 0·20 μCi of progesterone-4-^{14}C was added for the second time to the medium of tissue cultures and on the 23rd day the medium was removed and analysed for progesterone metabolites and cells fixed *in situ* for electron microscopy and for mitochondrial counting

Medium (Pharmaceutical Manufacturer, Orion Oy, Helsinki). Adrenocorticotrophic hormone (Cortrophine, pig ACTH, Organon) was added to the culture medium in the amount of 0·1 U per ml every day for six days from the 17th day of cultivation up to and including the 22nd day. The medium was changed every fifth day.

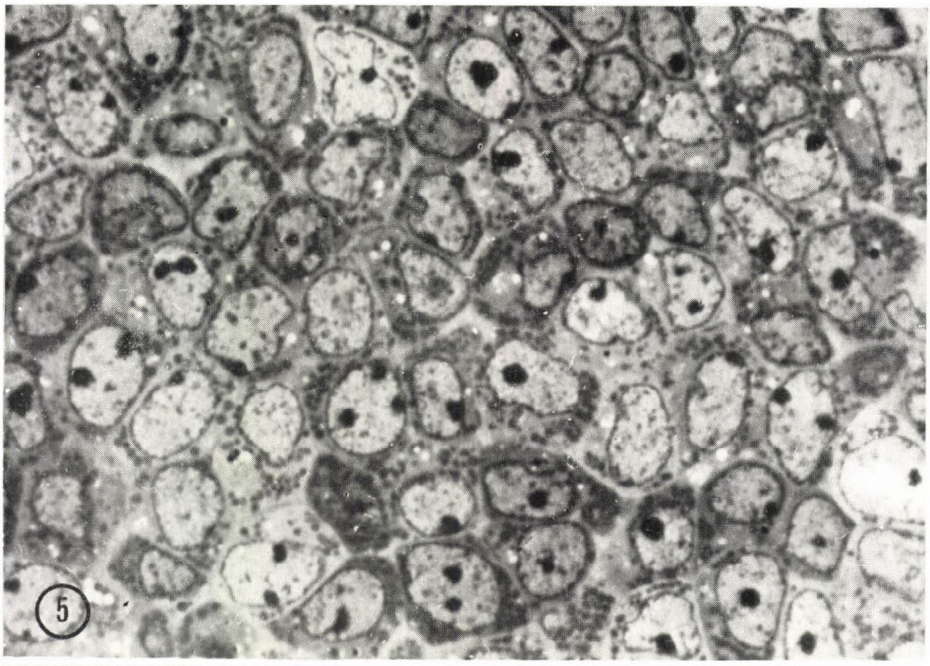
The substrate. Progesterone-4-^{14}C (The Radiochemical Centre, Amersham) with specific activity of 69 μCi per mg, was purified on TLC (chloroform : acetone, 9: 1) and dissolved in a propylene glycol-ethanol mixture (v/v 95 : 5) at the concentration of 1 μCi per 200 μl. 40 μl of this solution, equivalent to 0·20 μCi (445×10^3 DPM) of progesterone-4-^{14}C (about 3 γ of progresterone) was used for a single culture and added to the culture medium by an 'Agla' micrometer syringe having an accuracy of $\pm$ 0·1 μl. As schematically presented in Fig. 1, progesterone-4-^{14}C was first added on the 16th day of cultivation and after 24 h the medium was removed and used for the first steroid analysis (control value). New medium was added and on the 22nd day of cultivation progesterone-4-^{14}C was added the second time to the medium; after 24 h of cultivation, on the 23rd day the medium was removed and analysed for progesterone metabolites (second steroid analysis, experimental value). Cells were fixed for electron microscopy. Tissue cultures were carefully studied during experiments and before fixation by phase contrast microscopy.

Electron microscopy. Cultures were fixed *in situ* in 2·5 per cent glutaraldehyde in Hanks' BSS adjusted to pH 7·2 at the beginning of fixation and postfixed in 1 per cent osmium tetroxide with phosphate buffer (Millonig) or in 1 per cent osmium tetroxide in Hanks' BSS. After fixation they were dehydrated in graded series of ethyl alcohol and embedded *in situ* in a mixture of Epon 812 (Shell Chemical Corp., New York) and Araldite 6005 (Ciba, Basel). Thick sections were stained with toluidine blue for light microscopy and thin sections were cut with Sorval MT-1 ultramicrotome



Figs 2–3. Monolayer colony of cortical cells of foetal rat adrenals cultivated in tissue culture for 23 days. Vertical section stained with toluidine blue. Bars in Fig. 2 present area in Fig. 3 with high power objective. Note the bottom surface of culture (arrows). Horizontal line in Fig. 3 presents the section plane in Fig. 4 which has been used in mitochondrial counting. Note in Fig. 3 thin sheets of fibroblasts in both sides of cortical cell monolayer. ×170 and ×1,700, respectively

Fig. 4. Horizontal section of the monolayer of cortical cells in tissue culture of foetal rat adrenals cultivated for 23 days. ×340



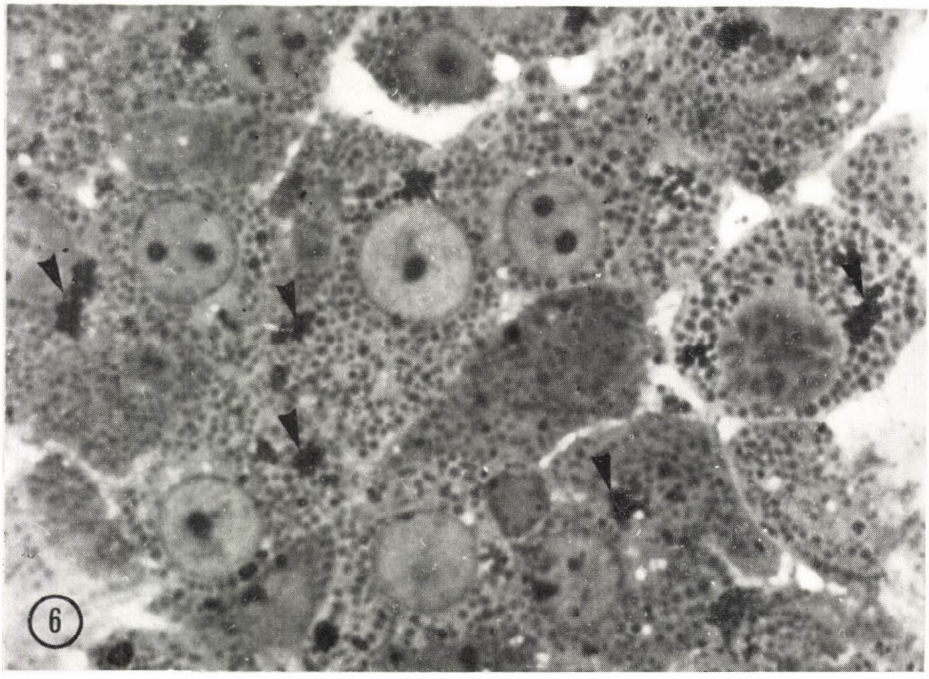


Fig. 5. Horizontal section of cortical cells in monolayer colony in tissue culture of foetal rat adrenals cultivated for 23 days in the absence of ACTH. Stained with toluidine blue. Mitochondria are very easily visualized as grey granules. × 1,700

Fig. 6. Horizontal section of cortical cells in monolayer colony in tissue culture of foetal rat adrenals cultivated for 23 days and stimulated with ACTH for 6 days. Note the grey granules as mitochondria and some black lipid granules (arrows). Note also the enlargement of cell size and nucleus. × 1,700

using glass knives. The sections were stained with 0·2 per cent lead citrate for 20 sec (Venable and Coggeshall, 1965). Electron micrographs were made at original magnifications of 1,500–30,000 with a Hitachi electron microscope.

Mitochondrial counting. The number of mitochondrial profiles in randomly sampled cortical cells was counted in horizontal sections (Figs 4–6). The section plane was chosen as presented in Figs 2—3 and it was approximately 7 μ from the lower surface of the cortical cell colony which was covered by thin fibroblastic sheets on both sides. Mitochondria were counted only in cortical cells which had in the section plane nucleus and nucleolus. Approximately 500 cortical cells in five colonies were counted both in control and ACTH-treated cultures. Mitochondria were easily visualized in toluidine blue stained thick plastic sections, and the nature of the granules was established electron microscopically using thin sections followed by thick sections. Occasionally there were very few lysosomes found in the cytoplasm of cortical cells which could not be differentiated from the mitochondria in thick sections.

Steroid analysis. Chromatography. Thin layer chromatography (TLC) was carried out on a 0·25 mm layer of Silica Gel G (Merck). Radioactive progesterone metabolites were separated by the techniques described by Pesonen and Saure (1969). Multiple (repeated) and bidimensional TLC-techniques were also used according to Lisboa (1963, 1966). Solvent systems used in TLC were slightly modified (Kahri et al., 1970).

Extraction, purification, separation and identification of metabolites of progesterone-4-^{14}C were carried out using techniques described earlier (Kahri et al., 1970). The final characterization of the radioactive progesterone metabolites was carried out by derivative formation with subsequent chromatographies on TLC and by recrystallization to constant specific activity as described earlier (Kahri et al., 1970).

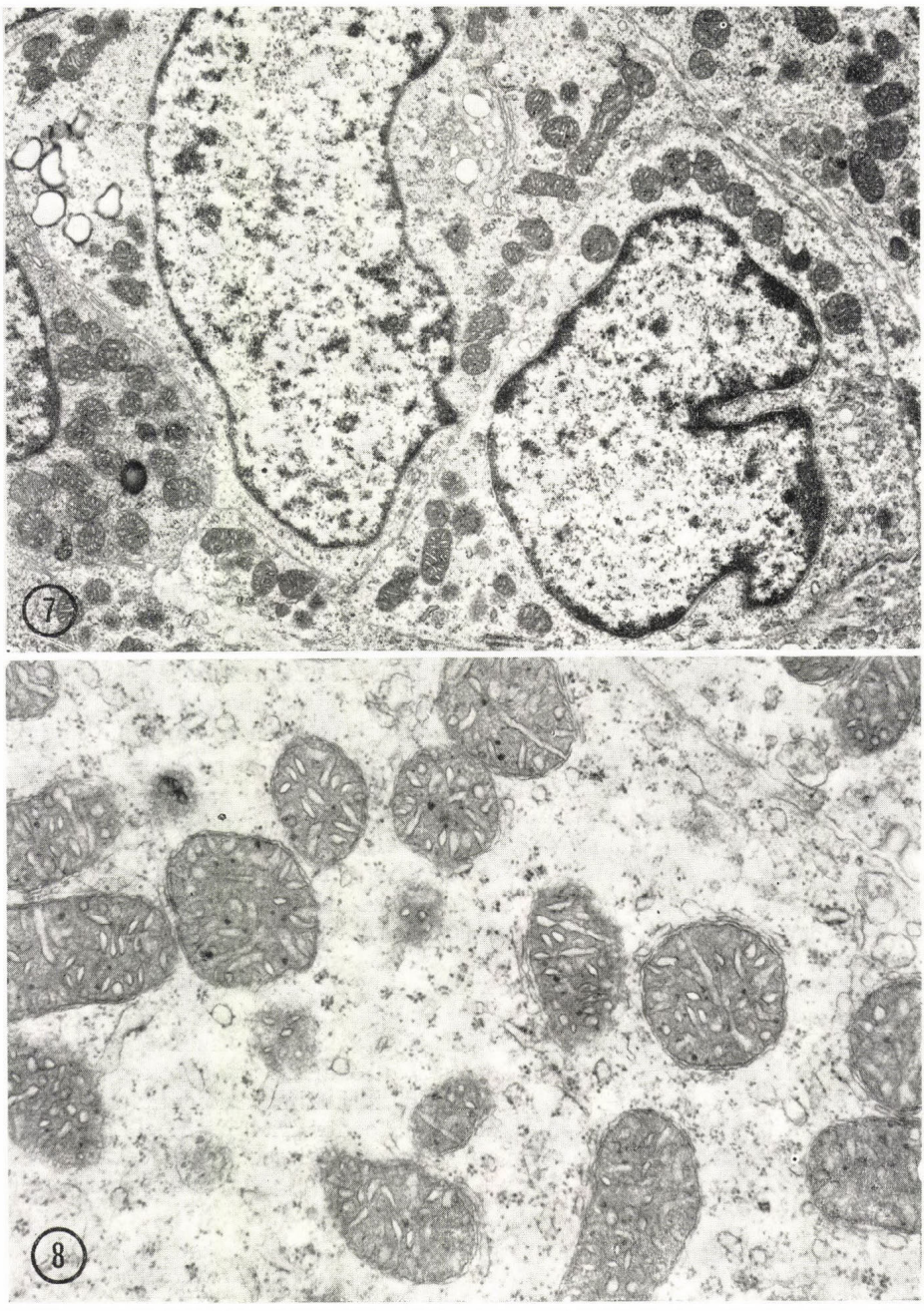
Cortical cells of fœtal rat adrenals in tissue culture have an ultrastructure of zona glomerulosa cells (Kahri, 1966, 1968, 1970) (Figs 7 and 8). During 6 days' ACTH stimulation they differentiate into zona fasciculata cells (Fig. 9), and the most specific affect of ACTH is the transformation of zona glomerulosa mitochondria, which have tubular or lamellar internal membranes (cristae), into zona fasciculata mitochondria with vesicular inner membranes (600 Å vesicles) (Fig. 10) (see also Kahri, 1966, 1968, 1970; Kahri et al., 1970).

The mean number of mitochondrial profiles in a horizontal section of cortical cells in tissue culture of fetal rat adrenals in the absence of ACTH was 24·8 $\pm$ 0·55 (mean $\pm$ S.E.). During ACTH stimulation there is a very significant increase in the number of mitochondrial profiles. The mean number of mitochondrial profiles in a horizontal section of cortical cells after ACTH stimulation is 90·6 $\pm$ 1·77 (Table 1). The mean number of

TABLE 1

Number of mitochondrial profiles in a horizontal section of cortical cells in the presence or absence of ACTH in tissue culture of foetal rat adrenals

	n	Mean	S.E.	*p*
Control	499	24·8	$\pm$0·55	
ACTH	498	90·6	$\pm$1·77	0·001



Figs 7–8. Cortical cells in tissue culture of foetal rat adrenals cultivated for 23 days in the absence of ACTH. Note the typical ultrastructure of zona glomerulosa cells and the mitochondria which have tubular or lamellar internal membranes (cristae). ×9,500 and ×34,800, respectively

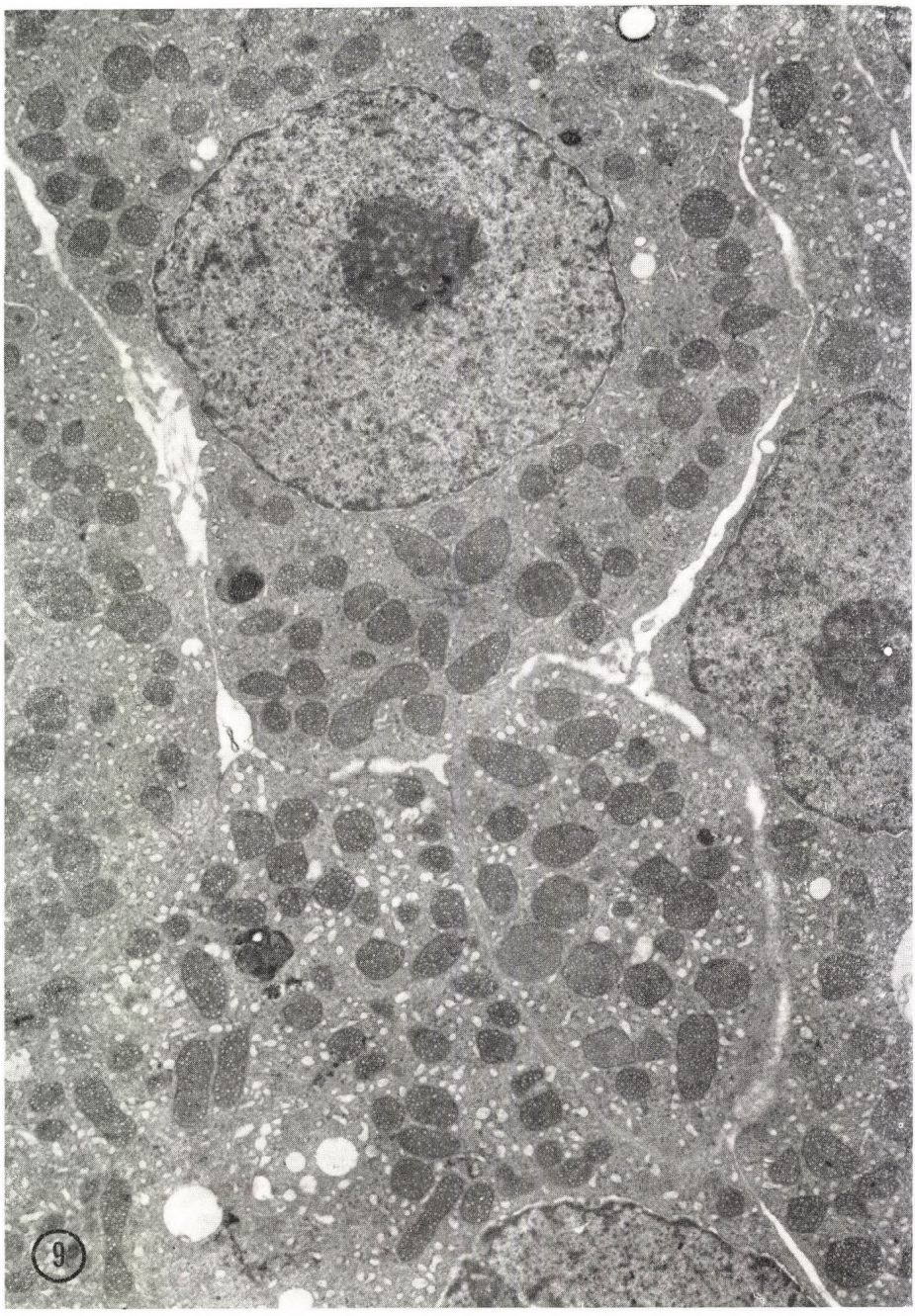


Fig. 9. Cortical cells in tissue culture of foetal rat adrenals cultivated for 23 days and treated with ACTH for six days. Note the increase in the number of mitochondria and transformation of the inner membranes of mitochondria into 600 Å vesicles. ×9,300

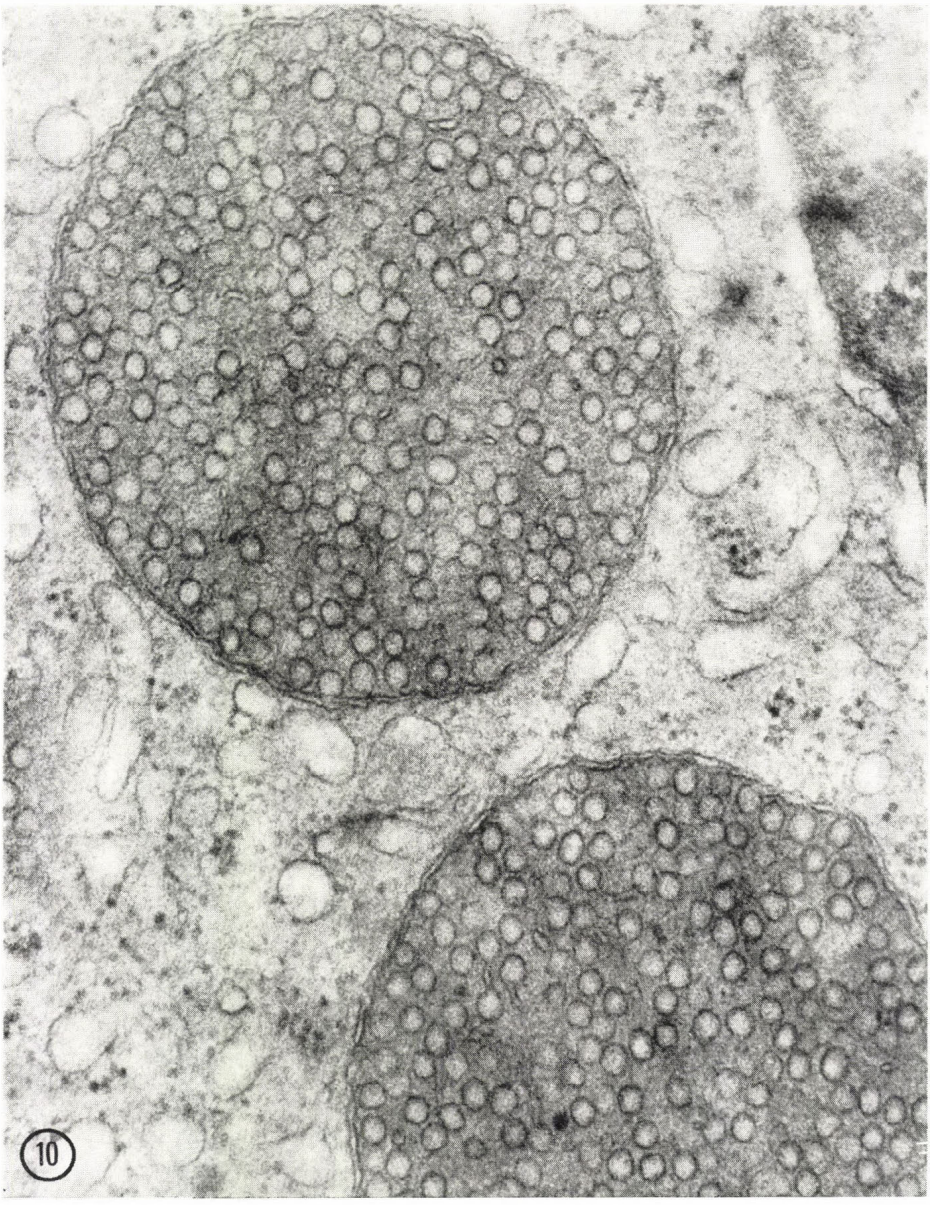


Fig. 10. Mitochondria in cortical cell treated with ACTH for 6 days. Cultivated for 23 days. Note the development of 600 Å vesicles in the matrix of mitochondria. × 72,200

mitochondrial profiles in cortical cells in control cultures varies between 20·0–35·2, and in ACTH treated cortical cells 69·4–113·2 in different colonies (Table 2). There was a significant difference between the mean number of mitochondrial profiles in cortical cells in each separate colony of the ACTH treated culture and the corresponding value in each colony of the control culture (t-test) (Table 3).

TABLE 2

Number of mitochondrial profiles in a horizontal section of cortical cells in different colonies of a tissue culture of fœtal rat adrenals in the presence or absence of ACTH (n = number of counted cells)

	No. of colony	n	Mean	S.E.
Control	1	111	35·2	± 3·3
	2	62	23·7	± 3·0
	3	174	20·0	± 1·5
	4	36	23·4	± 3·9
	5	116	22·9	± 2·1
ACTH	6	124	81·3	± 7·3
	7	101	113·2	±11·3
	8	121	96·0	± 8·7
	9	53	96·6	±13·0
	10	994	69·4	± 6·9

TABLE 3

Comparison of statistical significance (t-test) of mean number of mitochondrial profiles of cortical cells in separate colonies in ACTH treated culture with the corresponding values in cortical cells of each colony of control culture

		Control				
		1	2	3	4	5
ACTH	6	0·001	0·001	0·001	0·001	0·001
		14·28	17·31	14·21	14·69	11·56
	7	0·001	0·001	0·001	0·001	0·001
		14·48	15·60	13·22	14·69	13·15
	8	0·001	0·001	0·001	0·001	0·001
		25·08	26·79	22·94	24·81	22·72
	9	0·001	0·001	0·001	0·001	0·001
		11·23	12·01	10·18	11·44	10·30
	10	0·001	0·001	0·001	0·001	0·001
		19·89	21·43	18·20	20·20	18·01

Following TLC I (system 1) six to seven radioactive peaks were recorded by scanning on TLC plate in all analyses (Fig. 11) (Kahri et al., 1970). Figure 11 shows this typical distribution pattern of radioactivity in one of the analyses and the further identification of these fractions. The first fraction appeared to be appreciably more polar than corticosterone and aldosterone in the same system, indicating more than two hydroxyl groups incorporated in the original progesterone molecule. No efforts have been made, however, to identify this metabolite (see Kahri et al., 1970). The

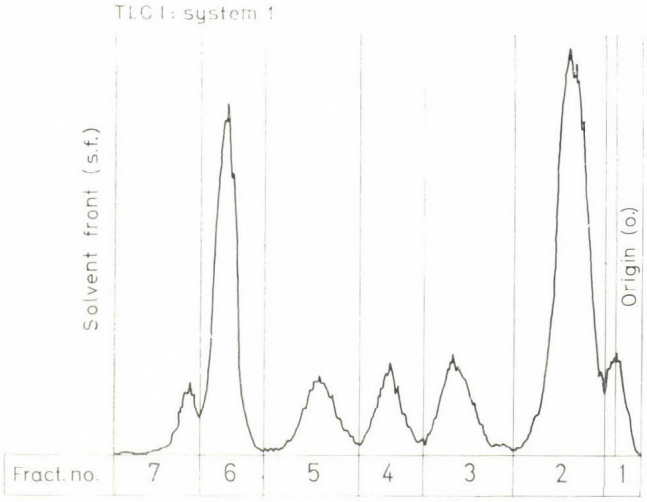


Fig. 11. A scanning record after TLC I showing a seven peak distribution pattern of radioactivity extracted from one tissue culture. P2 = corticosterone + 18-OH-DOC, P3 = 11β-hydroxyprogesterone + one unknown metabolite, P4 = DOC + 20 α-hydroxyprogesterone, P5 = unknown, unspecific metabolite, P6 = progesterone

TABLE 4

Progesterone-4-^{14}C metabolism in tissue cultures of fetal rat adrenals in the absence of ACTH, analysed on the 17th and 23rd days of cultivation

Fraction	*n*	Mean cpm	S.E.	*p*
P2				
Control 16	9	6·030	± 309	
Control 23	9	3·870	± 280	0·001
P3				
Control 16	9	7·435	± 636	
Control 23	9	11·423	± 456	0·001
P4				
Control 16	9	7·240	± 301	
Control 23	9	9·184	± 876	0·05
P5				
Control 16	9	5·491	± 333	
Control 23	9	5·385	± 240	NS
P6				
Control 16	9	13·823	±1760	
Control 23	9	5·377	± 220	0·001
Total count				
Control 16	9	42·164*	± 780	
Control 23	9	36·737	±1373	0·01

* 0·20 μCi of progesterone-4-^{14}C and not inactivated serum.
P2 = corticosterone + 18-OH-DOC; P3 = 11β-hydroxyprogesterone + one unknown metabolite; P4 = DOC + 20 α-hydroxyprogesterone; P5 = unknown, unspecific metabolite; P6 = progesterone.

second fraction contained corticosterone and 18-OH-DOC (18-hydroxy--deoxycorticosterone), the main steroids produced by rat adrenals. The third fraction contained 11β-hydroxyprogesterone and one unknown metabolite of progesterone. The fourth fraction contained DOC (deoxycorticosterone) and 20α-hydroxyprogesterone. The fifth fraction was an unknown metabolite of progesterone and not specific for adrenals (unpublished results). The sixth fraction was a progesterone fraction. Occasionally there was also a seventh fraction. Because of its high apolarity on TLC, it was mainly considered an artefact, especially as it was not recovered in all incubations (see also Kahri et al., 1970).

Incorporation of radioactivity into different fractions in the medium of tissue cultures of fetal rat adrenals on the 17th and 23rd days of cultivation in the absence of ACTH is presented in Table 4. There was a significant decrease in the conversion of progesterone-4-^{14}C into corticosterone-^{14}C and 18-OH-DOC^{14}C (fraction 2) on day 23 compared with the control values of the same cultures on day 17.

Incorporation of radioactivity into different fractions in the medium of tissue cultures treated with ACTH is presented in Table 5. The distribution of progesterone-4-^{14}C metabolites by the 17th day are the control values and ACTH values show the distribution of metabolites after 6 days of ACTH stimulation at day 23 in the same cultures. There was a very signif-

TABLE 5

Progesterone-4-C^{14} metabolism in tissue cultures of foetal rat adrenals in the presence of ACTH, double analysed on the 17th and 23rd days of cultivation. Control value on day 17 in the absence of ACTH and ACTH value from the same cultures on day 23 after six days' stimulation with ACTH

Fraction	*n*	Mean cpm	S.E.	*p*
P2				
Control	5	6·584	±1007	
ACTH	5	14·224	± 834	0·001
P3				
Control	5	12·080	±2091	
ACTH	5	14·596	±3280	NS
P4				
Control	5	14·522	±1595	
ACTH	5	10·996	±2403	NS
P5				
Control	5	9·752	± 992	
ACTH	5	16·032	±3872	NS
P6				
Control	5	13·068	±3940	
ACTH	5	15·644	±2888	NS
Total count				
Control	5	60·690*	±3141	
ACTH	5	75·080	±3976	0·05

* 0·25 μCi of progesterone-4-^{14}C and heat inactivated serum.
P2 = corticosterone + 18-OH-DOC; P3 = 11β-hydroxyprogesterone + one unknown metabolite; P4 = DOC + 20 α-hydroxyprogesterone; P5 = one unknown, unspecific metabolite; P6 = progesterone.

icant increase in the capacity of conversion of progesterone-4-^{14}C into corticosterone-^{14}C and 18-OH-DOC-^{14}C (fraction 2).

It has been shown in the present study that during ACTH induced differentiation of cortical cells and mitochondria with simultaneous increase in the capacity of mirochondrial 11β- and 18-hydroxylation there appeared a significant numerical increase of mitochondrial profiles. This indicates that during ACTH induced increase of functional capacity of cortical cells now mitochondria have been formed. Several hypotheses have been put forward on the formation of mitochondria (see the review of Schatz, 1970): (1) mitochondrial formation from other cytoplasmic organelles, (2) *de novo* formation, (3) formation by growth and division. The present findings on the mitochondria in cortical cells during growth and division furnish no information as to how they are formed and how the numerical increase in mitochondrial profiles comes about. It is, however, evident that cortical cells after ACTH stimulation contain approximately 4 times more separate mitochondrial DNA molecules than untreated cortical cells if we assume that all mitochondria in undifferentiated cortical cells contain only one DNA molecule per mitochondrium. However, it seems to be evident that all the mitochondria are not monomeric with reference to the number of DNA molecules they contain. Some mitochondria may contain numerous DNA molecules. The present observation gives no answer to the question whether this ACTH induced increase in the number of mitochondria is caused by division of mitochondria with multimeric DNA into monomeric form or replication of mitochondrial DNA and division of mitochondria after synthesis of new mitochondrial DNA.

As presented here, ACTH induced significant increase in two mitochondrial steroidogenic enzymes, 11β- and 18-hydroxylases which is in good agreement with the ultrastructural transformation of the mitochondrial inner membranes. This confirms the results of our earlier work (Kahri et al., 1970) which has been done using slightly different cultivation and substrate incubation techniques. In our earlier work there was no replenishment of media during cultivation and, therefore, there was no mitosis in the cortical cells after two weeks of cultivation. In the present system cortical cells are continuously diving even during ACTH treatment. However, ACTH induced similarly in both systems the differentiation of mitochondria and an increase in the number of mitochondrial profiles in cortical cells. So it seems to be evident that mitochondrial growth and division is not linked to the cycle of cell division and synthesis of nuclear DNA. In the system which we used earlier there were also differences in substrate incubation. As presented in Fig. 12 (published earlier, see Kahri et al., 1970), there is an approximately 100 per cent increase in the conversion of progesterone-4-^{14}C (0·25 μCi of substrate incubated for 6 days in the presence and absence of ACTH) in ACTH treated cultures compared with the controls. It is very interesting that differentiation of mitochondria occurred after three days' stimulation preceding the significant elevation in the capacity of 11β- and 18-hydroxylation in cortical cells. We have done some preliminary observations on the effects of ACTH on the mitochondria of cortical cells of human fetal adrenals in tissue culture and found that after three days' stimulation with ACTH there appeared numerous DNA fibres in the mitochondria of the stimulated cells (unpublished results). This will support the hypothesis

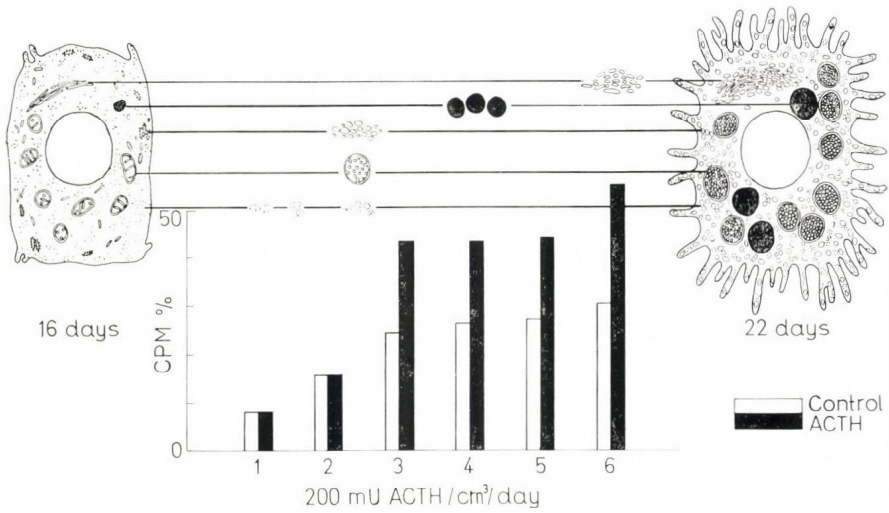


Fig. 12. Schematic representation of ACTH induced ultrastructural differentiation of cortical cells in tissue culture of foetal rat adrenals. Note the ACTH induced significant increase in the conversion of progesterone-4-^{14}C into corticosterone and 18-OH-DOC and transformation of mitochondria into zona fasciculata type (Kahri et al., 1970)

that mitochondrial DNA synthesis is probably stimulated earlier than the division of mitochondria occur, and the synthesis of mitochondrial DNA takes place at a very early stage of mitochondrial life cycle.

Numerical increase of mitochondria after ACTH treatment (if we assume that it indicates replication of mitochondrial DNA) indicates protein synthesis of cortical cell mitochondria, which is different from the synthesis of structural proteins (cristae) in the mitochondria. ACTH induced development of mitochondrial cristae in cortical cells have been found to be dependent on the mitochondrial protein synthesis (Kahri, 1970) (chloramphenicol sensitive). In further studies it has been shown that 11β-hydroxylase (Kahri and Milner, 1969; Kahri et al., 1971) and 18-hydroxylase (Kahri et al., 1971) are dependent on the mitochondrial protein synthesis (chloramphenicol sensitive). Stimulation of the synthesis of structural proteins (cristae) (Kahri, 1971) and dependence of synthesis of 11β- and 18-hydroxylases on the mitochondrial protein synthesis (Kahri et al., 1971) are dependent on nuclear DNA directed protein synthesis and cannot be stimulated with ACTH directly (Kahri, 1971, Kahri et al., 1971). Stimulation of mitochondrial DNA directed protein synthesis is mediated through cytoplasmic ribosomal protein synthesis and can be inhibited with cycloheximide (Kahri, 1971; Kahri et al., 1971). It has not been clarified as yet how the synthesis of mitochondrial DNA and the division of mitochondria is regulated and linked with the nuclear DNA directed protein synthesis.

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FINE STRUCTURE AND HORMONAL ACTIVITY OF INTACT AND CULTURED EMBRYONIC ADRENAL CELLS OF DIFFERENT SPECIES

by

A. Gyévai, B. Bukulya, K. Mihály, K. Szalay and E. Stark
INSTITUTE OF EXPERIMENTAL MEDICINE, BUDAPEST, HUNGARY

Until quite recently, data pointing to an ACTH producing capacity of the human foetal pituitary and of the corticosteroidproducing ability of the foetal adrenal were remarkably scarce, and rested on indirect evidence.

It became necessary, therefore, to try to answer the following questions: Can the embryonic pituitary produce ACTH and the adrenals produce corticosteroids? Does the adrenal respond to ACTH? A rational approach to these problems seemed to be the combined cultivation of pituitary and adrenal tissue.

Our earlier investigations on about 100 cultures showed that at the beginning of the second trimester of gestation the human foetal pituitary secreted ACTH and the adrenal produced glucocorticoids characteristic of the adult (Stark et al., 1965*a*).

The ability of the foetal adrenal to produce corticoids in tissue culture has been corroborated by Bloch et al. (1965). Our finding that the cultured pituitary is capable of secreting ACTH (Stark et al., 1965*b*) was substantiated by Fleischer and Rawls (1970) using radio-immunoassay.

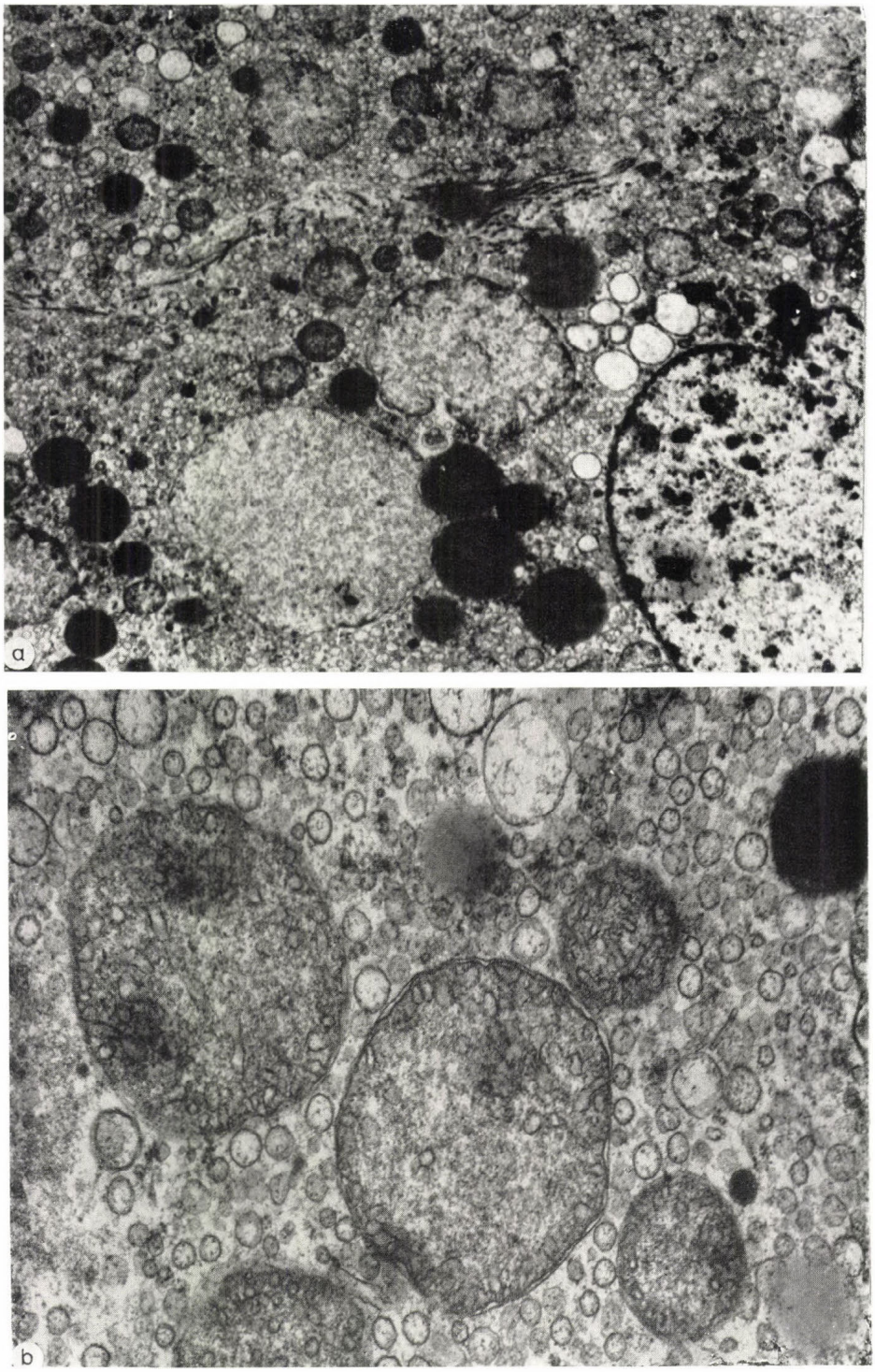
Subsequent experiments revealed that when the two organs were cultured together the corticosteroid content in the medium considerably decreased, or was nil, at various points of time, mostly after 8 to 12 days. Further experiments showed that coritcosteroid production declined because the ACTH production had ceased. The adrenal fragments retained their corticoid producing ability, since in response to exogenous ACTH corticosteroid production was restored. It was striking that production increased not 24, but only 72 h after addition of ACTH. This we interpreted as a sign of the cells losing their normal fine structure during cultivation, the recovery of which is a precondition of corticoidogenesis.

This interpretation obviously demanded a careful investigation of the relationship between the fine structure and the steroid biosynthetic activity of foetal adrenocortical cells.

Kahri (1966) showed that cultured cells grown for 16 days in the absence of ACTH lose their normal fine structure, which is gradually recovered when ACTH is added to the medium daily for 6 days; further, that the rate of corticosteroid production increases parallel with the rate of recovery.

HUMAN EXPERIMENTS

The present experiments were performed to examine the relationship between fine structure and hormonal activity of the adrenocortical cells.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The experimental procedure was as follows: Adrenals were cut into fragments. The fragments obtained from one of the adrenals in each foetus were placed into a 250 ml Falcon flask, and those from the contralateral adrenals into another one, on top of coagulated fowl plasma and embryonic juice. The culture medium consisted of an 8 : 2 mixture of TC 199 and human serum. The fragments in one set of the flasks were used for control purposes and were cultured without adding ACTH to the medium. The other group were grown in a medium to which 100 mU per ml ACTH had been added at various intervals. The amount of corticosteroids in the medium was determined by fluorometry. ACTH was added to the medium in some instances daily, in others every second or third day, since it had been observed earlier that the cultures continued to produce corticosteroids for long periods of time.

Table 1 presents a typical experiment (F_{100}) showing the amounts of corticosteroids produced at different times of growth by control adrenals and by adrenals treated with ACTH.

TABLE 1

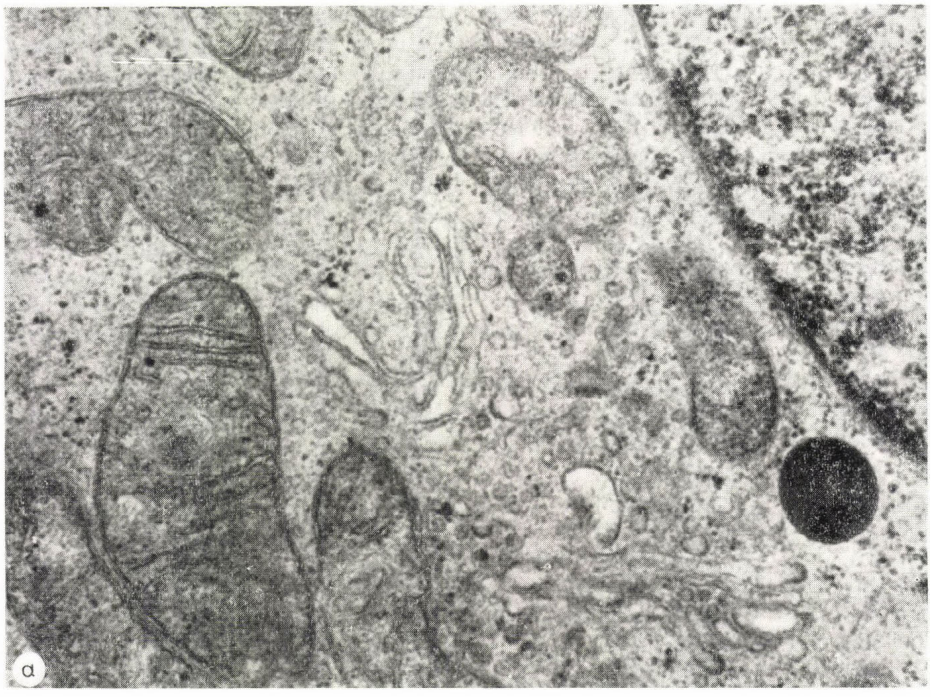
Corticoid production of human foetal adrenal tissue culture
F_{100}

Days after explantation	Control corticoids, μg/24 h	ACTH treated
2	1·4	1·9
3	0·9	4·3
4	no medium change	
5	no medium change	
6	0·4	20·1
7	no medium change	
8	0·4	24·7
9	no medium change	
10	1·2	13·2
11	no medium change	
12	no medium change	
13	0·8	10·2
14	no medium change	
15	no medium change	
16	no medium change	
17	0·6	5·5
18	no medium change	
19	no medium change	
20	0·6	5·8

Table 2 (F_{101}, F_{102}) shows the corticosteroid production by cultures processed for electron-microscopic examinations. Respectively on the 6th and 7th days of growth these cultures were fixed, inside the flasks, in glutaraldehyde, embedded in Epon and counterstained with uranyl acetate. To the

←

Figs 1*a*, *b*. Electron micrographs of human foetal adrenals in the second trimester of gestation. ×8,340 and ×34,000, respectively



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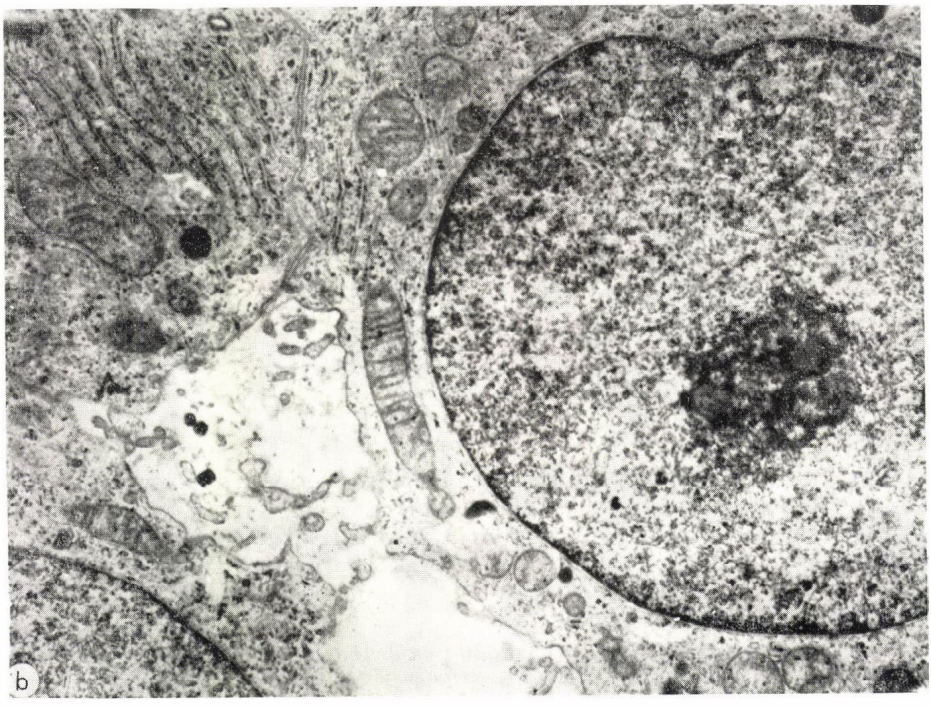

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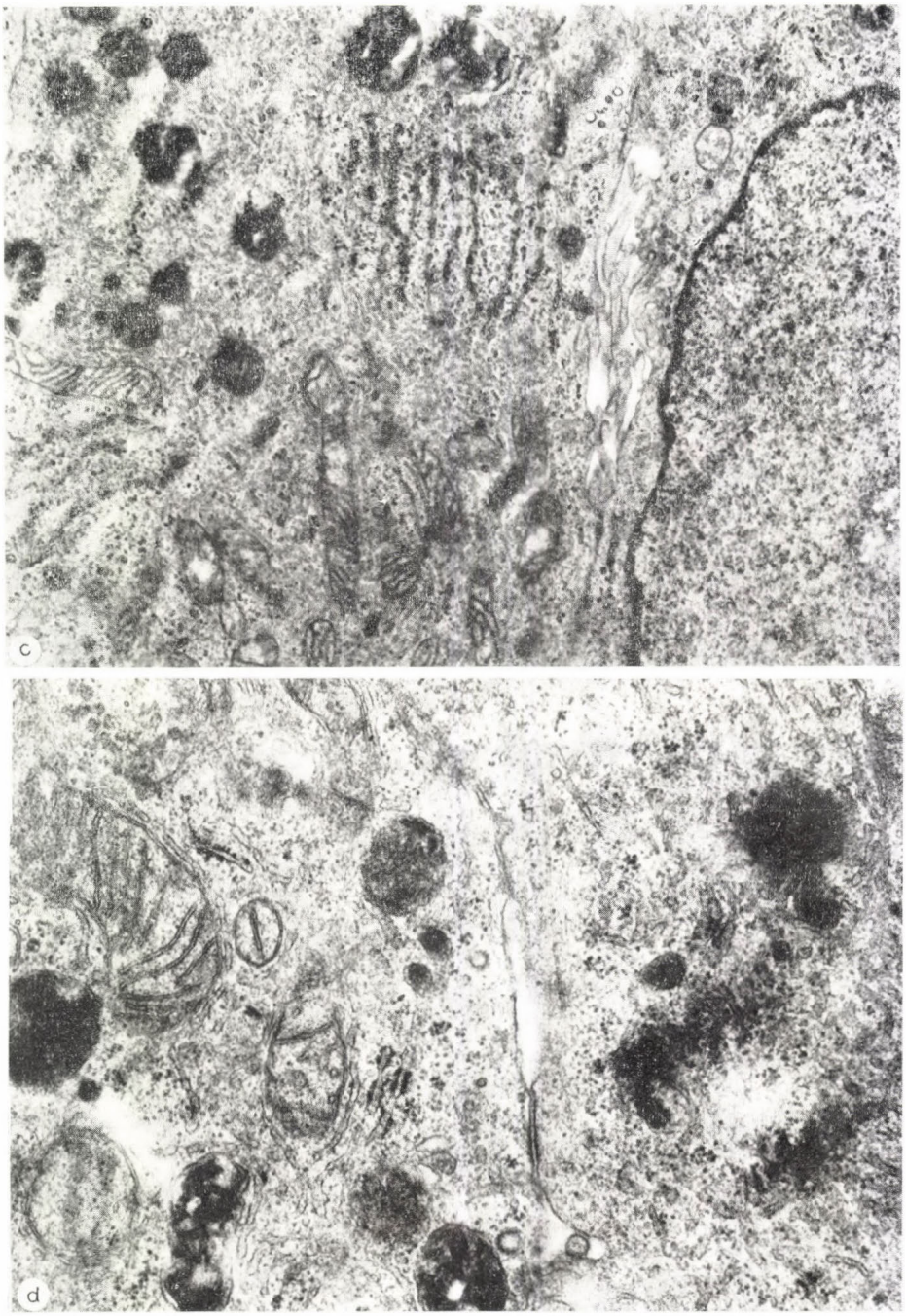
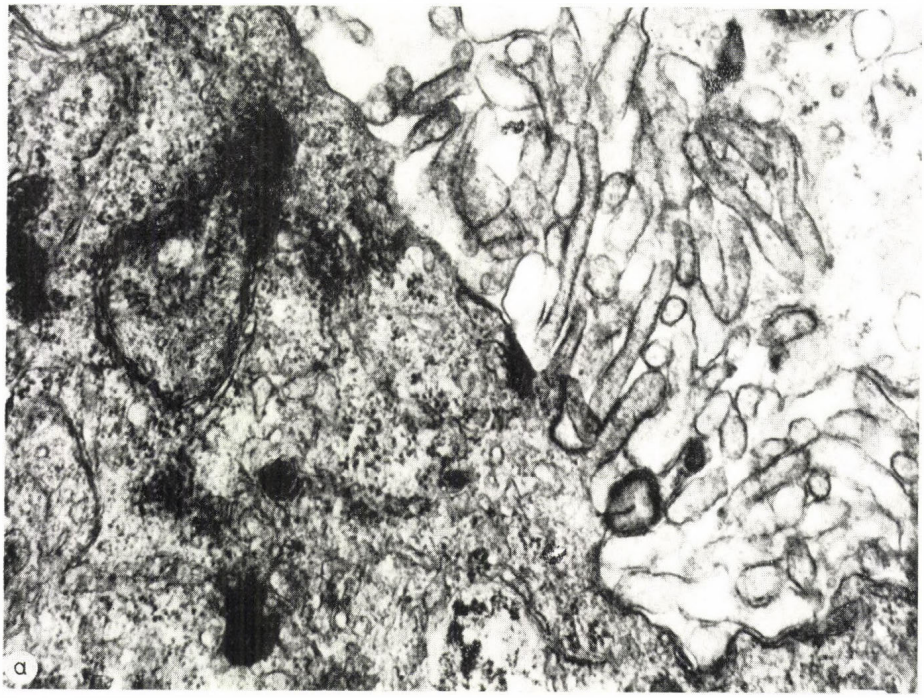


Figs 2*a*, *b*, *c*, *d*. Electron micrographs of human foetal adrenals cultured for 6 or 7 days without ACTH. For details see text. × 14,800; ×23,700; ×12,500; ×9,600, respectively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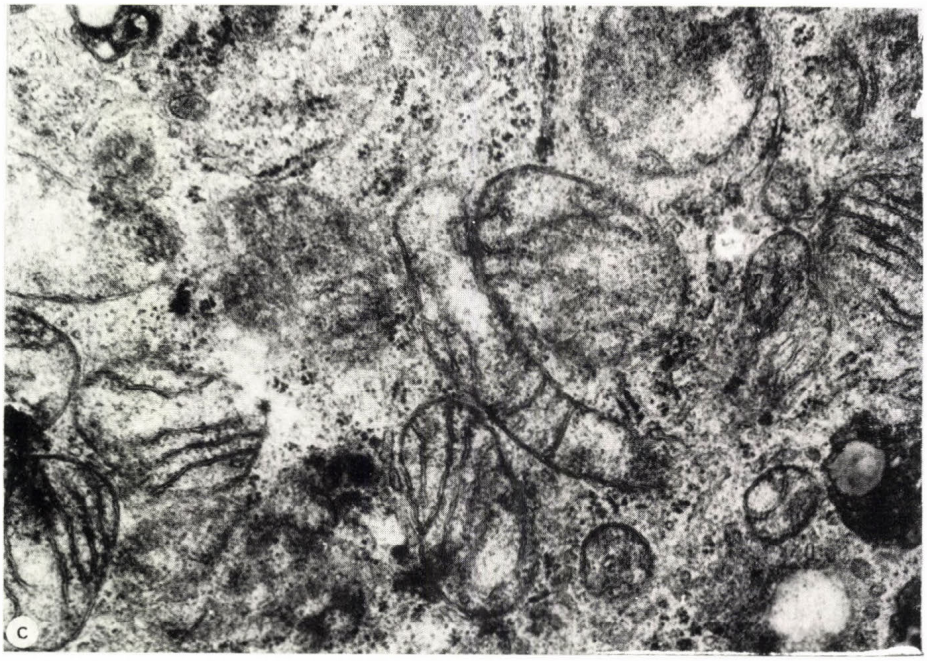

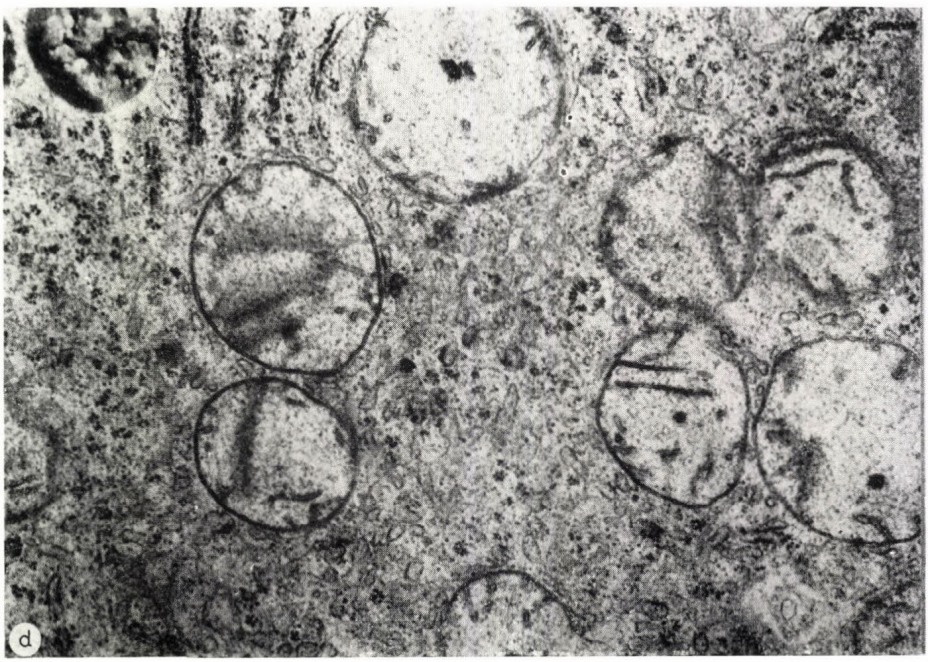

Figs 3*a*, *b*, *c*, *d*. Electron micrographs of human foetal adrenals cultured for 6 or 7 days with ACTH. For details see text. ×35,300; ×36,100; ×32,300; 21,600, respectively

TABLE 2

*Corticoid production by cultures processed for electron-microscopic examinations on the 6th and 7th day of growth**

Days after explantation	Control corticoids, μg/24 h	ACTH treated corticoids, μg/24 h
	F_{101}	
2	4·1	2·8
3	no medium change	
4	no medium change	
5	1·0	9·6
6	no medium change	
7	1·9	26·1
	F_{102}	
2	5·5	9·2
3	no medium change	
4	no medium change	
5	1·0	13·0
6	1·5	14·9

* All cultures processed for electron microscopy were fixed inside the Falcon flasks in 2·5 per cent glutaraldehyde and 1 per cent OsO_4, embedded in Durcapan ACM and examined in a JEM 6AS electron microscope.

6-day cultures ACTH was last added 24 h before fixation, and to the 7-day cultures 48 h before fixation.

The fine structure of the starting material (0-time material) agreed with the picture described by Johannisson (1968) for the human foetal adrenal of comparable age (Fig. 1*a*). The cells were characterized by spherical nuclei, very dense chromatin near the nuclear membrane, and prominent, smooth-surfaced endoplasmic reticulum (Fig. 1*b*). The mitochondria were spherical and tubulovesicular. In and near the large Golgi complex osmiophilic granules were seen. A few microvilli were seen covered by the cell membrane.

The fine structure of the adrenal cultured for 6 or 7 days without ACTH differed from that of the 0-time material. Less nuclear chromatin was seen, which was evenly distributed (Fig. 2*a*). The mitochondria were elongated and mostly cristate (Fig. 2*b*). Most of the rough-surfaced endoplasmic reticulum was vesicular, the rest, tubular. Smooth-surfaced reticulum was seen only sporadically and was found to be the vesicular type. Groups of electron-dense lipid droplets could be observed (Fig. 2*c*). Desmosomes were present between the cells (Fig. 2*d*). The cell membranes covered but a few microvilli.

There seem to be few characteristic marks which distinguish the fine structure of the adrenals treated with ACTH 48 h before fixation on the 7th day from the fine structure of the control. The most striking feature, however, is an increase in the number of microvilli in the ACTH treated cells (Fig. 3*a*). Besides, there is a slight decrease in the number of mitochondria, although their shape and structure are the same as in the controls, except for an occasional reduction in electron density (Fig. 3*b*). The endoplasmic reticulum is in some areas rough-surfaced and vesicular or, at places,

lamellar, while in other areas it is smooth-surfaced and vesicular. Lipids are fewer and less dense.

It is less difficult to find differences in fine structure between cells treated with ACTH 24 h before processing and untreated control material. More mitochondria are seen, but in shape and structure they agree with those in the controls (Fig. 3*c*). The endoplasmic reticulum is rough-surfaced or smooth-surfaced, and mostly vesicular. In some areas the endoplasmic reticulum is lamellar (Fig. 3*d*). Lipid droplets are sparse. Membrane-covered microvilli are numerous.

What has been said so far permits the conclusion that the fine structure of the human foetal adrenal grown *in vitro* differs from that seen in the 0-time material. In the adrenal cells cultured in ACTH-free medium dedifferentiated mitochondria and endoplasmic reticulum appear as early as on the 6th or 7th day of growth. And what is remarkable, we observed an essentially similar fine structure in the cells of adrenal cultures treated with ACTH which produced corticoids in large amounts.

RAT EXPERIMENTS

Adrenals derived from 17- to 21-day-old rat embryos were cultured, fundamentally by Kahri's method. The fragmented adrenals were placed into Falcon flasks on top of coagulated fowl plasma and embryonic juice. The culture medium was a mixture of Melnick's solution and calf serum. After explantation, air containing 5 per cent CO_2 was forced into the flasks, which were then sealed off.

In these experiments we used the same setup as in the case of human foetal adrenals. In each of 17 Falcon flasks the 40 adrenals of 20 embryos were cultured; ACTH was added to the medium of 6 cultures, while the rest were cultured without ACTH. The medium was not changed during cultivation. Cultures were processed on the 4th, 8th and 14th days of culturing.

TABLE 3

Corticosterone production of foetal rat adrenal tissue culture (μg/culture)

Days after explantation	No. of culture	Control	No. of culture	ACTH treated
4	1	4·4	1_a	31·5
	2	5·2	2_a	23·7
	3	3·5	3_a	12·6
8	4	2·5	4_a	9·9
	5	4·4	5_a	42·5
	6	4·4	6_a	47·0

In Table 3 the corticosterone production in the cultures is shown. There is a striking difference in the corticosterone amount between the control and the ACTH treated cultures.

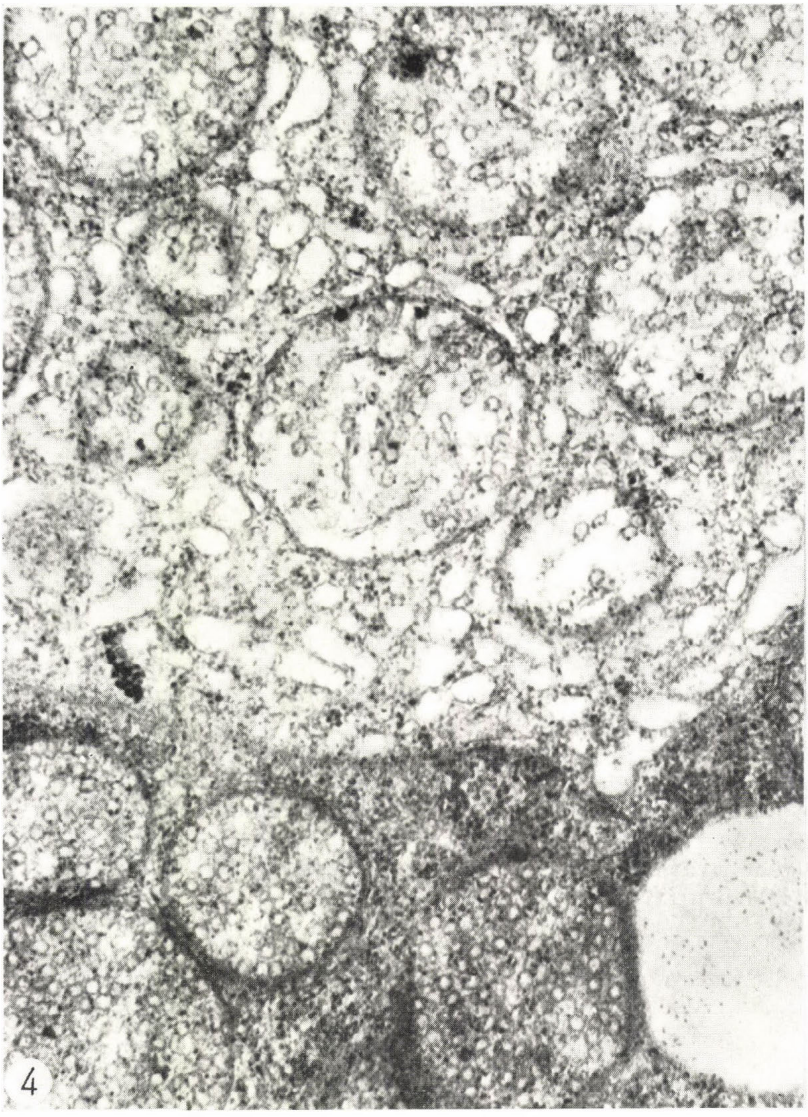

Fig. 4. Electron micrograph of a 21-day-old embryonic rat adrenal × 35,300

The fine structure of the adrenals from 17- to 21-day-old rat embryos was described by Idelman (1966) and also in an earlier work of our own. The cells were found to be characterized by agranular endoplasmic reticulum and vesicular mitochondria (Fig. 4).

The adrenals grown for 4, 8, and 14 days without ACTH did not show any important differences in fine structure. They differed, however, from the fine structure seen in the 0-time material in that the mitochondria

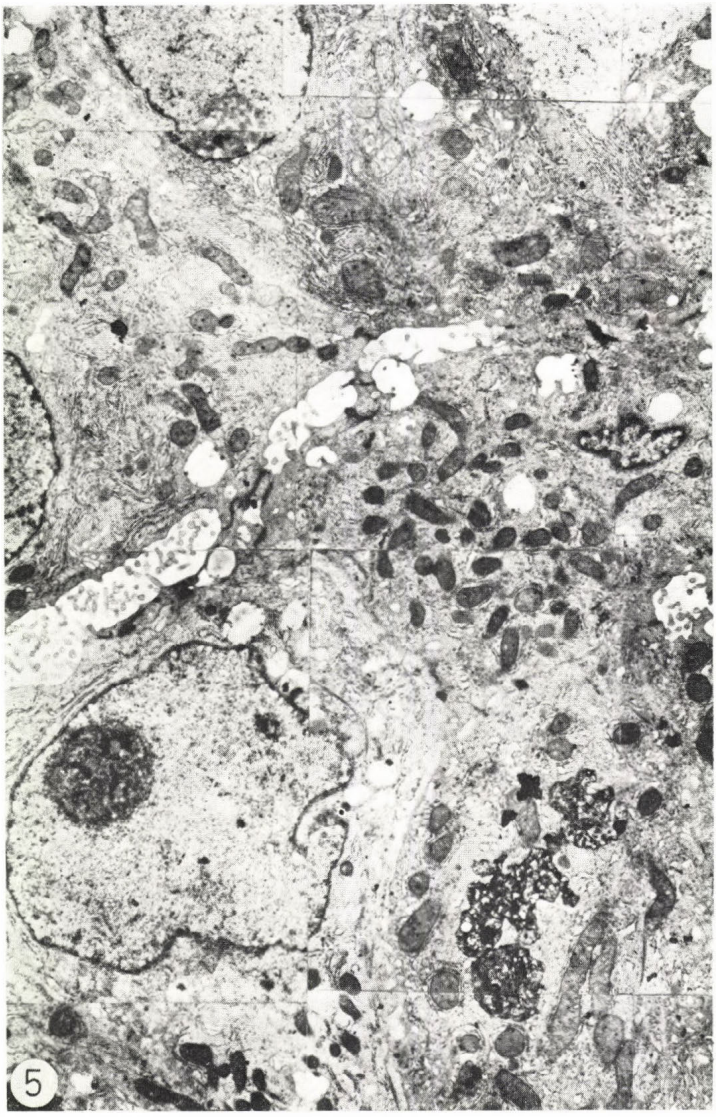

Fig. 5. Electron micrograph of an embryonic rat adrenal cultured for 4 days without ACTH. ×2,700

became cristotubular, the endoplasmic reticulum turned rough-surfaced and lamellar, and more microvilli appeared (Fig. 5). The medium of ACTH treated cultures contained considerable amounts of corticosterone. Independent of the day when the cultures had been processed (4th, 8th, or 14th day), the fine structure showed the following common features (Fig. 6): more mitochondria and endoplasmic reticulum, though these were similar in structure to those of cells cultivated without ACTH; i.e. the mitochondria

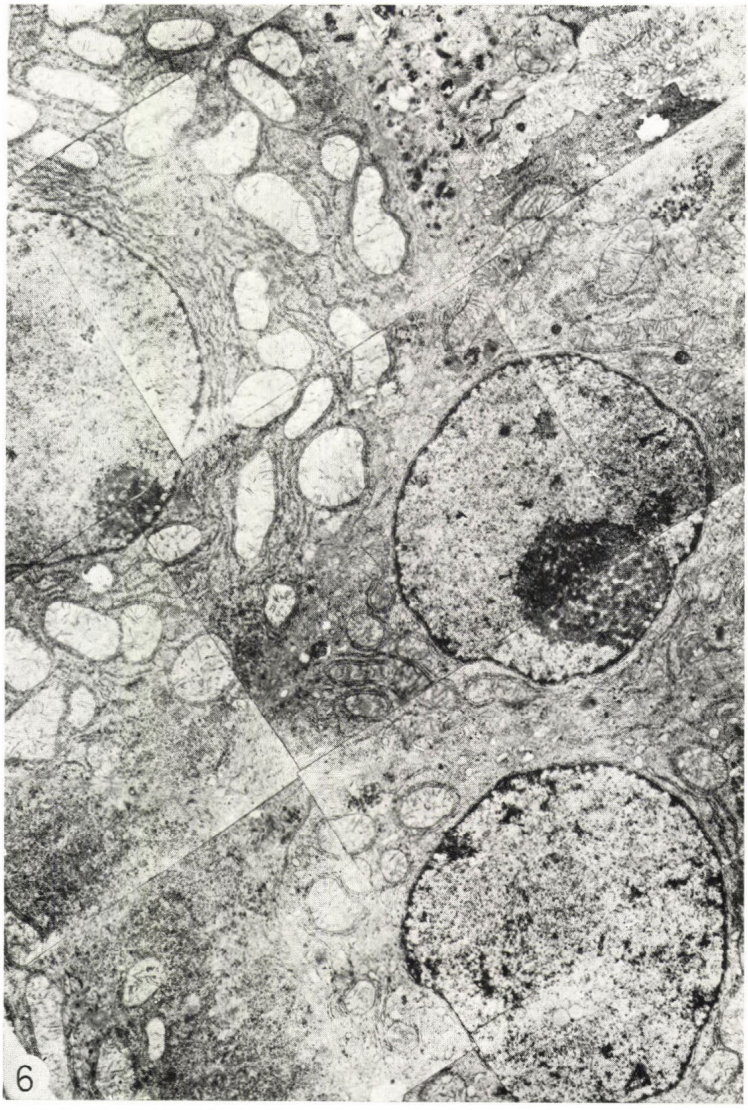

Fig. 6. Electron micrograph of an embryonic rat adrenal cultured for 4 days with ACTH. ×2,600

were cristotubular, and the endoplasmic reticulum was rough-surfaced and lamellar. Fewer lysosomes were present and considerably more microvilli were seen than in the controls.

How is one to comment on these findings?

The fine structure of the human foetal adrenal at the time of explantation was characterized by the presence of vesicular mitochondria, smooth-surfaced reticulum, and a few microvilli. Thus it agreed with the fine struc-

ture of the cells of the zona fasciculata. In the cells grown for 6 or 7 days in ACTH-free medium, the mitochondria were cristotubular and the endoplasmic reticulum rough-surfaced, i.e. resembled some earlier stage. These cells produced corticosteroids in barely measurable amounts. The fine structure of the adrenal fragments grown for the same number of days in ACTH containing medium, differed little from that of the controls, and yet produced corticosteroids in substantial amounts.

As stated before. when ACTH was added to cultures that had ceased to produce corticosteroids, hormone production started again on the 3rd day and significant amounts could be measured. The assumption that the adrenal cells regain their responsiveness when the organelles regain their normal structure, i.e. when they are transformed into zona-fasciculata-type cells was not supported by our findings. In a work using the method of Kahri (1966), Milner and Villee (1970) likewise found that foetal adrenals that had been cultured in ACTH-free medium produced corticosteroids as late as on the 3rd day after ACTH had been added to the medium. Recently Milner (1971) has reported that embryonic rat adrenal cells cultured for 16 days do not respond to ACTH within the first 60 min whereas cells pretreated with ACTH do. However, the reponse to ACTH was practically the same in the two cultures on the 3rd day after a single exposure to ACTH. Milner does not mention what the fine structure was like at the time of the response to ACTH. But Kahri observed complete regeneration of the fine structure of dedifferentiated adrenal cells after treatment with ACTH daily for six consecutive days. From this, one is forced to assume that in Milner's work, the response given on the 3rd day to a single dose of ACTH added to the adrenal cells previously cultured in an ACTH-free medium came about when the fine structure was dedifferentiated. In the light of our own findings and of data in the literature we have formed the opinion that in tissue culture the human foetal adrenal cells are capable of responding to ACTH with corticosteroid production at least for some time, irrespective of whether they agree with or differ from the cells in the zona fasciculata in their fine structure.

We have arrived at a similar conclusion with embryonic rat adrenal cultures. Cells which at explantation had a characteristically zona fasciculata fine structure showed signs of dedifferentiation as early as the fourth day of growth irrespective of whether or not the medium contained ACTH. The fact that fragments cultured in the presence of ACTH produce many times as much corticosterone as the same amount of control fragments can only be interpreted to indicate that the corticoidogenic action of ACTH is independent of its fine structure maintaining effect.

The results obtained in the present experiments would by no means justify the conclusion that the adrenals respond unconditionally with corticosteroid production in the absence of a normal fine structure characteristic of the zona fasciculata. We can only refer to the established fact that ACTH is capable of maintaining the corticoidogenic activity of the cultured adrenal cells without preserving their normal fine structure.

In the experiments described so far the cells which possessed a normal fine structure at the time of explantation lost it during cultivation and yet they responded to ACTH with corticosteroid production.

The potentiality to produce corticosteroids as well as responsiveness to

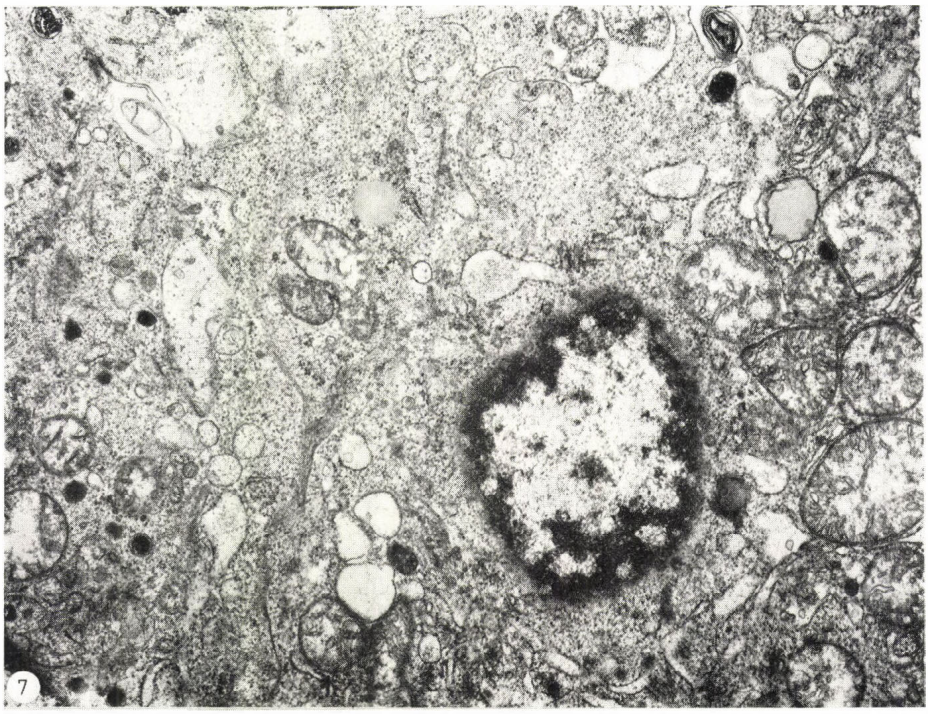

Fig. 7. Electron micrograph of a 2 cm (crown-rump length) cat adrenal. ×17,500

ACTH preceding the development of the fine structure in the zona fasciculata was shown by Stark et al. (in press). Adrenals of cat embryos measuring 2 to 13 cm were removed, one of them was prepared for morphological examination and the other one incubated with or without ACTH by the method of Saffran and Schally (1955). The embryonic cat adrenals are capable of producing corticosteroid very early during their development (Table 4). Figure 7 shows the fine structure of the adrenal from a 2 cm cat

TABLE 4

Effect of ACTH on the corticoid production of embryonal cat adrenal in vitro (corticoids, μg per 100 mg)

Crown-rump length cm	Control	100 mU ACTH per 100 mg adrenal
1·7	no data	4·8*
2·0	74·4	211·2*
3·5	no data	12·6
6·0	3·1	8·8
9·0	11·2	18·5
10·0	4·7	18·7
11·0	3·0	7·9
12·0	7·2	16·3
13·0	20·4	35·3

* ng/2 adrenals.

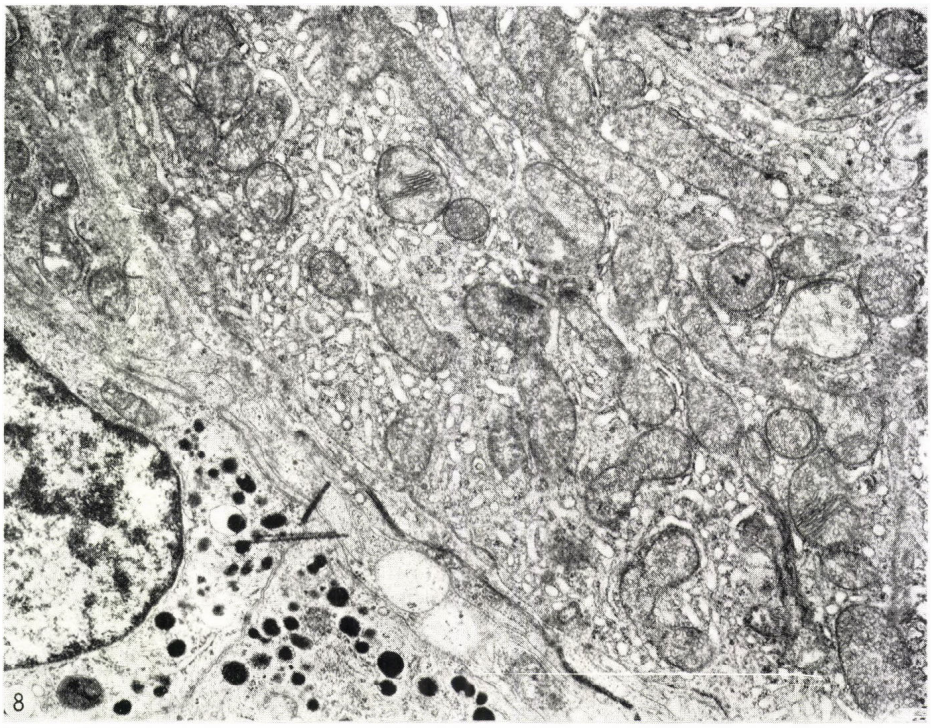

Fig. 8. Electron micrograph of a 6 cm cat adrenal. × 13,200

embryo and Fig. 8 from a 6 cm cat embryo. It was found that the cells produced corticosteroids and responded to ACTH before the fine structure characteristic of the zona fasciculata had developed.

As a summary, cultured embryonic adrenals possessing fully developed fine structure retain for some time their ability to produce corticosteroids even when that structure—above all in the mitochondria and the endoplasmic reticulum—shows signs of returning to earlier stages. The ability of the embryonic adrenals to respond to ACTH precedes during ontogenesis the definite fine structure characteristic of the zona fasciculata.

In tissue culture the metabolic processes needed for specific cellular activity run their courses irrespectively of whether or not the fine structure, on which these processes are generally believed to depend, corresponds to the picture characterizing the normal cells.

ACTH is known to be the specific stimulus of adrenal activity and the factor mainly responsible for the maintenance of structure. It would be, however, an unwarranted simplification to suppose that the presence or absence of fine structure of the adrenal depends exclusively on ACTH.

The tissue culture method may be a useful means of detecting other factors which in cooperation with ACTH, would be responsible for the maintenance of the fine structure.

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Symp. Biol. Hung. 14, pp. 89–96 (1972)

THE FINE STRUCTURE OF EMBRYONIC RAT ADRENALS CULTURED *IN VIVO*

by

B. Bukulya, I. Ökrös, and E. Stark
INSTITUTE OF EXPERIMENTAL MEDICINE, BUDAPEST, HUNGARY

The fine structure of adrenocortical cells in adult rats is well known (Yamori et al., 1961; Nishikava et al., 1968; Yoshimura et al., 1968; Szabó et al., 1970). We have some knowledge of its changes following various experimental interventions, such as hypophysectomy (Lever, 1956; Sabatini and De Robertis, 1962; Volk and Scarpelli, 1966; Szabó et al., 1967; Yoshimura et al., 1968; Nussdorfer and Mazzochi, 1970), ACTH treatment (Lever, 1956; Aschworth et al., 1959; Yamori et al., 1961; Szabó et al., 1967, 1970; Yoshimura et al., 1968; Nussdorfer and Mazzochi, 1970), exposure to various kinds of stress (Yamori et al., 1961; Harumiya, 1966; Yoshimura et al., 1968; Seki and Sekiyama,, 1969; Szabó et al., 1970) and pharmacological procedures (Schwarz and Suchowsky, 1963; Volk and Scarpelli, 1964; Yoshimura et al., 1968; Dzsinich et al., 1969; Horváth et al., 1969; Seelig and Rennels, 1969; Nussdorfer and Mazzochi, 1970). Few studies have, however, been devoted to the development of the fine structure in the mammalian and human foetal adrenal cortex. Systematic investigations have been carried out by Idelman (1966) and ourselves (Bukulya et al., 1967) in rat embryo adrenals, and by Johannisson (1968) in human foetal adrenals.

It has been established that on the 17th day of gestation the rat cortical cells contain all the fine structural elements seen in the adult animal.

The present report is meant to give an account of the effect which ACTH exerts on the development of the fine structure under different experimental conditions.

A few pictures will be submitted in the following demonstrating, on the basis of earlier work from our laboratory, the development of the fine structure in the rat embryo adrenal cells producing glycocorticoids.

The developmental changes in the embryo are best followed in the mitochondria and endoplasmic reticulum. Other organelles will not be considered, unless they reveal fundamental discrepancies. The adrenals were fixed with glutaraldehyde and osmium tetroxide in 0·1 M sodium cacodylate buffer. Because this buffer elicited myelinization and shrinkage, an 0·05 M buffer was used for adrenals younger than 17 days. Ultrathin sections were counterstained with uranyl acetate and lead citrate.

Figure 1 shows that, most interestingly, up to the 13th day of gestation the cortical and medullary cells are conspicuous for their mixed presence in the anlage of the adrenal and for their great likeness in fine structure. The mitochondria are cristate and the endoplasmic reticulum is granulolamellar. The only difference is that the cortical cells contain slightly more

osmiophilic (lipid) droplets and that a few dense-core granules occur in the medullary cells.

On the 15th day of gestation (Fig. 2) the mitochondria are enlarged, become spherical, and a few tubules and vesicles appear in them. Most of the endoplasmic reticulum is still lamellar and granular but, at places, protrusions and vesicle-like processes are visible. At and near the protrusions few ribosomes are present on the outer surface of the lamellae. No ribosomes can be observed either on the vesicle-like processes or on the vesicles themselves. In our view, this morphology provides evidence of incipient transformation of the cristate into tubulovesicular mitochondria and of the granulolamellar endoplasmic reticulum into an agranular vesicular one.

On day 17 (Fig. 3), mainly the mitochondria and the endoplasmic reticulum can be observed to undergo vesicular transformation. On day 18, the fine structure (Fig. 4) resembles that in the adrenocortical cells of the mature rat.

During the perinatal period (Fig. 5) the mitochondria, the endoplasmic reticulum and the other organelles grow richer in structure, but do not alter in character, i.e. they increase in amount and size. The matrix of the mitochondria shows higher density, the vesicles increase in number, the Golgi apparatus expands and displays several lamellae, more vesicles are seen in its area, more lysosomes are observed and the lipid droplets turn pale.

It remained to investigate the non-genetic factors that may be involved in the development of the structure of the rat adrenocortical cells; further, the time when during ontogenesis such factors become active. The obvious thing to do was to fall back on ACTH as a non-genetic factor.

First the method of *in vivo* cultivation in the anterior chamber of the eye was adopted because we thought this technically undemanding method the most suitable for the cultivation of isolated organs, and also because it seemed reasonable to assume that ACTH would reach the adrenals transplanted into the anterior eyechamber.

Transplantation was carried out by the usual technique. In the figures below transplants will be shown in which vascularization could not be seen even under the light microscope. Successful hypophysectomy was checked by light microscopy.

It is common knowledge that in the adrenalectomized, dexamethasone-treated and hypophysectomized animals ACTH secretion increases, decreases, and ceases, respectively.

→

Fig. 1. Portion of an adrenocortical cell of a 13-day-old normal rat embryo. The mitochondria are cristate (cm) and the endoplasmic reticulum is granulolamellar (ger). l, lipid droplet. ×34,800

Fig. 2. Portion of an adrenocortical cell of a 15-day-old normal rat embryo. The mitochondria are enlarged, and spherical, and a few tubules and vesicles are in them (tvm). Most of the endoplasmic reticulum is still lamellar and granular (ger) but, at places, protrusions and vesicle-like processes (vlp) are visible. ×34,800

Fig. 3. Portion of an adrenocortical cell of a 17-day-old normal rat embryo. Mainly the mitochondria (tvm, vm) and the endoplasmic reticulum (arrow) can be observed to undergo vesicular transformation. ×34,800

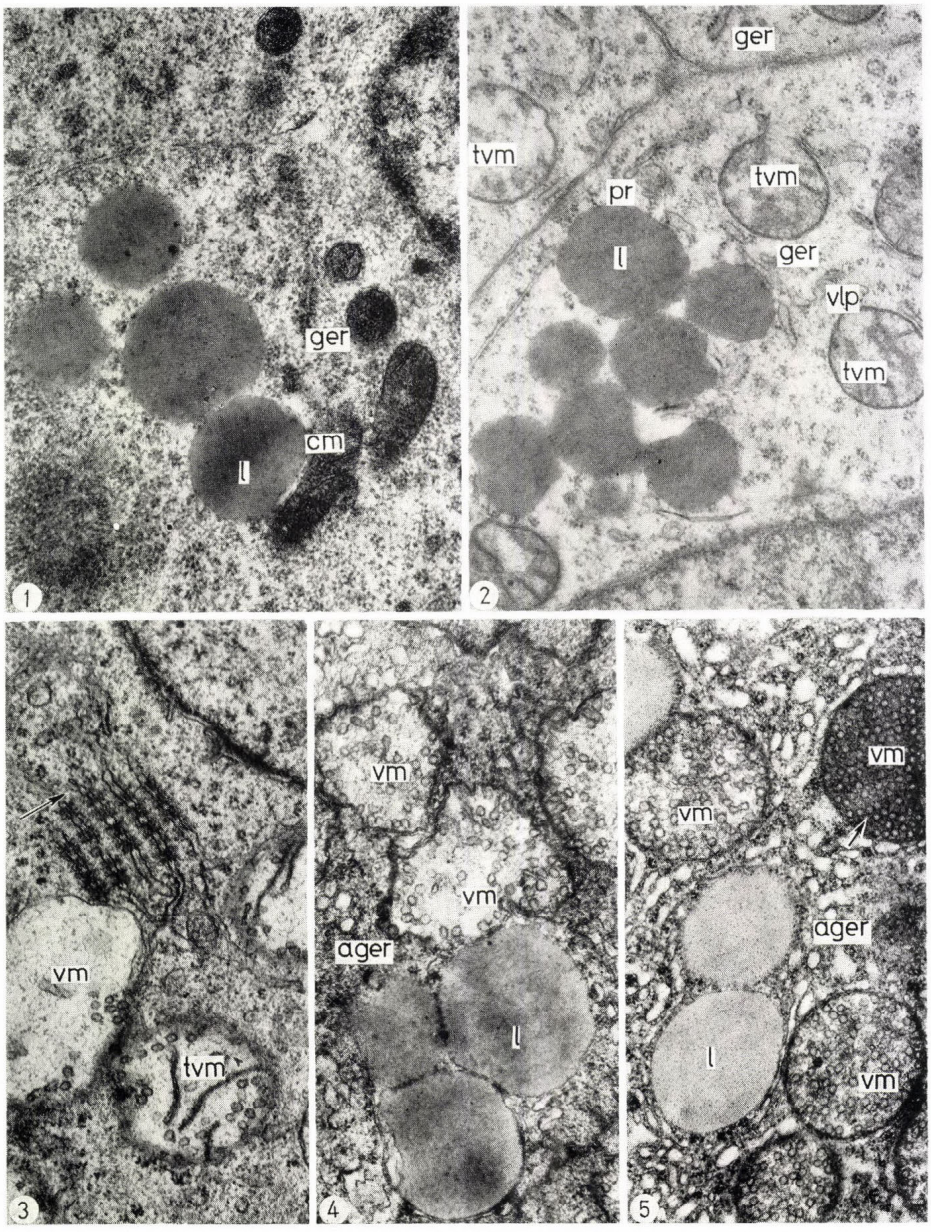

Fig. 4. Portion of an adrenocortical cell of an 18-day-old normal rat embryo. The fine structure resembles the adrenocortical cells of the mature rat; ager, agranular endoplasmic reticulum. ×34,800

Fig. 5. Portion of an adrenocortical cell of a 21-day-old normal rat embryo. The mitochondria (vm), the agranular endoplasmic reticulum (ager), and other organelles are rich in structure, but did not alter in character. The matrix (arrow) of the mitochondria shows higher density, vesicles increase in number. ×34,800

Therefore, adrenals derived from rat embryos of different ages were transplanted into the anterior eyechamber of host animals.

Our starting point was that if we transplanted the adrenal of a 13-day-old embryo into the anterior eyechamber of a rat—at a time when the mitochondria and the endoplasmic reticulum were not yet transformed—and cultured it in the chamber, we might experience considerable transformation within days. To our surprise, no transformation whatsoever could be observed even after cultivation for 21 days (Fig. 6). Therefore, it may be assumed that the ACTH of the host animal does not reach the transplanted adrenal cells, or is ineffective. What seems more probable is that transformation of the adrenocortical cells is not ACTH-dependent.

For this reason we cultured, for various periods, adrenals derived from 15-day-old embryos; at this age transformation has already begun. In the 1-day-old culture (Fig. 7), we observed that the endoplasmic reticulum, the mitochondria, and other organelles—practically the whole cellular fine structure—was almost the same as that in the adrenocortical cells of the normally developing 21-day-old embryo.

In the 4-day and 6-day cultures (Figs 8, 9) the fine structure is similar to that in the adult adrenal that shows hyperfunction after treatment with formalin or ACTH.

The morphological appearence of the fine structure pointing to hyperfunction is even more pronounced after cultivation in adrenalectomized host animals (Fig. 10). In such cases, particularly after cultivation for 5 days, 'malformed' mitochondria appear, such as can be observed after long-term treatment with large doses of ACTH in the adult animal.

When host animals are treated with dexamethasone or hydrocortisone daily for six days, then the fine structure in the adrenal of the 15-day-old rat embryo is similar to that in the untreated host animals (Fig. 11), though the mitochondria are smaller, and the matrix is more electron dense, and their structure turns vesicular. The smooth-surfaced endoplasmic reticulum is vesicular, lipids are scarce, and the Golgi apparatus consists of only a few lamellae. The nuclear surface is uneven and the nucleus is electron dense. Hardly any microvilli are present.

Even these few experiments suffice to evidence that the way ACTH secretion is influenced in the host animal will be invariably reflected by the fine structure of the transplanted embryonic adrenal.

The role of ACTH in the formation of the adrenocortical cells is confirmed by the experiments of *in vivo* culturing in the anterior eyechamber of hypophysectomized host animals.

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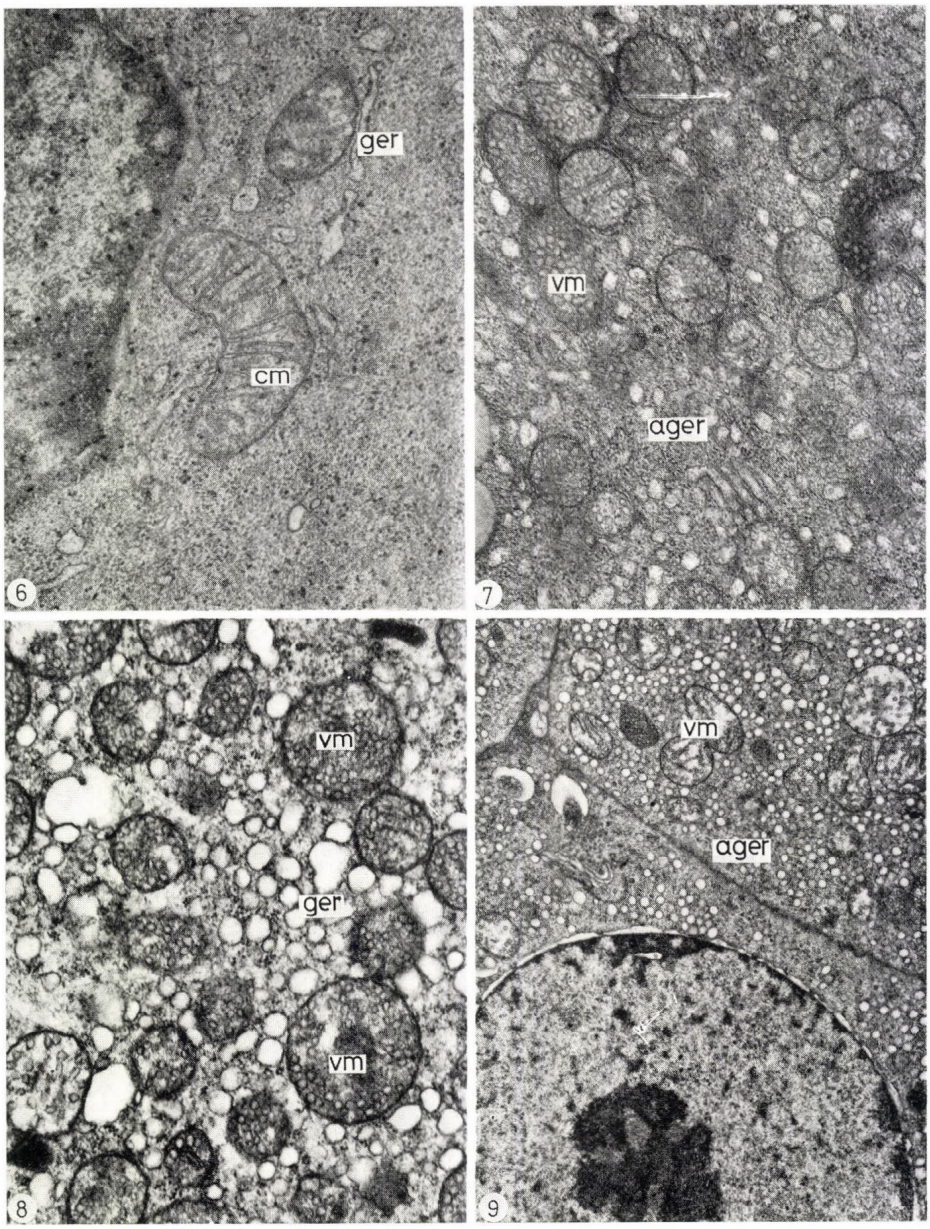
Fig. 6. Portion of an adrenocortical cell of a 13-day-old normal rat embryo cultured for 21 days in the anterior eyechamber of a normal adult rat. No transformation whatsoever could be observed even after cultivation for 21 days. cm, cristate mitochondria; ger, granular endoplasmic reticulum. ×50,000

Fig. 7. Portion of an adrenocortical cell of a 15-day-old normal rat embryo cultured for 1 day in the anterior eyechamber of a normal adult rat. The agranular endoplasmic reticulum (ager), the mitochondria (vm) and other organelles—practically the whole cellular fine structure—is almost the same as in the adrenocortical cells of the normally developing 21-day-old embryo. ×37,200



Figs 8 and 9. Portion of an adrenocortical cell of a 15-day-old normal rat embryo cultured for 4 or 6 days in the anterior eyechamber of a normal adult rat. The fine structure is similar to that in the adult adrenal that shows hyperfunction after treatment with formalin or ACTH. $\times 34{,}800$ and $\times 12{,}400$, respectively

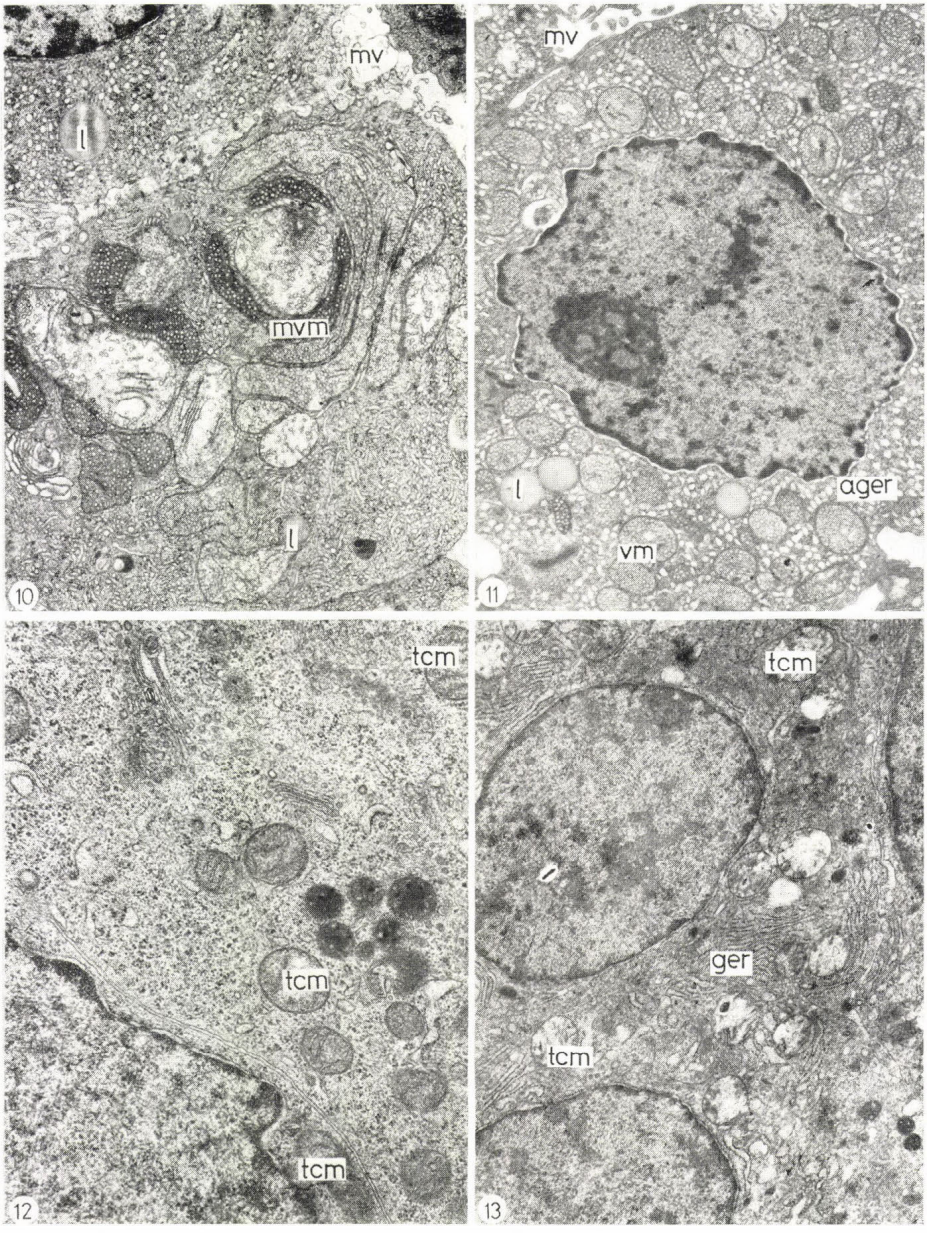


Fig. 10 .Portion of an adrenocortical cell of a 15-day-old normal rat embryo cultured for 6 days in the anterior eyechamber of an adrenalectomized adult rat. In such cases there arise 'malformed' mitochondria (mvm). mv, microvilli; l, lipid droplet. × 13,700

Fig. 11. Portion of an adrenocortical cell of a 15-day-old rat embryo cultured for 6 days in the anterior eyechamber of an adult rat treated with dexamethasone for 6 days. The fine structure is similar to that in the untreated host animal, though mito-

The fine structure of the 1-day culture of the adrenal derived from a 15-day-old embryo is less adrenal in character (Fig. 12) than the original material.

The 6-day culture is already dedifferentiated (Fig. 13) to the point where its fine structure does no longer resemble the adrenal cortex.

The fine structure of the adrenal cells of the 15-day-old embryo transplanted into the anterior eyechamber of normal rats undergoes transformation as *in situ*, moreover differentiation is accelerated if ACTH secretion is increased by adrenalectomy. There will be no differentiation, however, in the hypophysectomized animal, and only protracted differentiation when the animal is treated with corticosteroids. The present work definitely points to the important role played by ACTH in the transformation of the fine structure during ontogenesis.

It would be difficult to conceive another interpretation for the fact that interventions, interfering with ACTH secretion in the host animal, cause parallel changes in the differentiation of the fine structure of the transplanted adrenal.

Nevertheless, these experiments do in no way exclude the role of other factors in the differentiation of the fine structure.

*

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chondria (vm) are smaller, the matrix is more electron dense, and their structure is vesicular. The endoplasmic reticulum (ager) is agranular and vesicular. mv, microvilli. ×12,800

Fig. 12. Portion of an adrenocortical cell of a 15-day-old rat embryo cultured for 1 day in the anterior eyechamber of a hypophysectomized adult rat. The fine structure of the adrenocortical cell is less adrenal in character than the starting material; tcm, tubulo-cristate mitochondria. ×34,800

Fig. 13. Portion of an adrenocortical cell of a 15-day-old rat embryo cultured for 6 days in the anterior eyechamber of a hypophysectomized adult rat. It is already dedifferentiated to the point where its fine structure no longer resembles the adrenal cortex. ger, granular endoplasmic reticulum. ×11,300

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ELECTRON-MICROSCOPIC STUDY OF SPONTANEOUS TRANSFORMATION OF LYMPHOCYTES AND MONOCYTES IN TISSUE CULTURE

by

I. Blazsek, A. Gyévai and A. Balázs
INSTITUTE OF EXPERIMENTAL MEDICINE, BUDAPEST, HUNGARY

The transformation capacity of lymphocytes and monocytes was assumed by several authors as early as the beginning of this century (Woodliff, 1964). The transformation of lymphocytes into macrophages has recently been proved by the experiments of Gough et al. (1965) and Chapman et al. (1967), the promonocyte–monocyte–macrophage transformation by those of van Furth et al. (1970) and van Furth and Diesselhoff-Den Dulk (1970), in short-term cultures. According to Sutton and others, in the course of cultivation for 6 to 24 h, monocytes are transformed into macrophages, later into epitheloid cells and multinucleated giant cells (Woodliff, 1964; Sutton and Weiss, 1966; Sutton, 1967). Gerber and Monroe (1968) found multinucleated giant cells and immature lymphoblastoid cells in human leukocytes cultured for 16 to 160 days. Formation of fibroblast-like cells in leukocyte culture has also been reported (Rappay et al., 1968).

In our study we intended to clarify: (*i*) which types of the mononuclear leukocytes are resistant to the given cultivation condition, and (*ii*) which new cells types resulting from spontaneous transformation, appear in addition to the resistant types in short term tissue cultures. As to the latter, we asked first of all whether the so-called 'PHA-blast' cells known in cultures stimulated with phytohaemagglutinin or other mitogens are formed via spontaneous transformation.

Leukocytes obtained by spontaneous sedimentation of the blood of healthy human donors were cultured in a medium containing 80 per cent Parker 199 and 20 per cent human serum of AB group. Cultures were processed for light and electron microscopy, 0, 24, 48, 72 and 120 h after explantation. Slides for light microscopy were stained with Giemsa solution. For electron microscopy the cells were fixed in glutaraldehyde and osmium tetroxide, embedded in Durcupan ACM; the sections were stained with uranyl acetate and lead citrate.

Light-microscopic investigation revealed that in the 'buffy coat' cultures the total cell count rapidly decreased between the 24th and 72th h and dropped to the quarter of the original in the 120th h. It can be seen in Table 1 that this results from the degeneration of the neutrophil granulocytes. At the same time the incidence rate of the lymphocytes and macrophage-type cells increased.

In the original material the following cell types, not belonging to the granulocyte cell line, could be seen in the electron microscope:

(1) Few large lymphocytes of young character (Fig. 1).

TABLE 1

Changes of the total cell count and the differential cell count in the 0, 24, 48, 72 and 120 h human leukocyte cultures

Cultivation h	Lymphocytes		Monocytes and macrophages	Neutrophil granulocytes	Eosinophil and basophil granulocytes	Cell count per ml
	small	medium and large				
0	68·7	20·3	11·0			
	28·7	8·1	4·6	57·3	1·3	$2{\cdot}2\times10^6$
24	67·5	20·5	12·0			
	34·1	11·6	6·4	46·2	1·7	$1{\cdot}8\times10^6$
48	46·3	31·7	22·0			
	38·1	26·1	18·1	13·8	3·9	$1{\cdot}1\times10^6$
72	33·5	40·0	26·5			
	30·2	38·0	23·8	4·2	3·8	$0{\cdot}7\times10^6$
120	28·4	39·0	32·6			
	25·4	36·6	30·0	5·5	2·5	$0{\cdot}5\times10^6$

(2) A greater number of medium-size lymphocytes, for which relatively well-developed nucleoli, many mitochondria, an underdeveloped Golgi apparatus, occasionally groups of lysosomes, poly- and first of all free ribosomes are characteristic (Fig. 2).

(3) The small lymphocytes prevailed. Several types could be distinguished: (*a*) Round cells with nuclei rich in heterochromatin and cytoplasm poor in organelles. These were similar to the small lymphocytes found in the thymic cortex. (*b*) Cells containing a relatively well-developed nucleolus but cytoplasm poor in organelles; sometimes phagosomes and autophagic vacuoles were seen in them. (*c*) Cells with nuclei containing relatively well-developed nucleoli and more abundant cytoplasm richer in organelles. They were generally characterized by the cytosome and a number of large mitochondria with a dense matrix.

(4) Several lymphocytes characterized by 1 to 4 lamellae of rough surfaced endoplasmic reticulum surrounding concentrically the nucleus. In our opinion they are transitional forms between lymphocytes of young character and typical plasma cells.

(5) Monocytes were frequently seen. In their kidney-shaped nucleus 1 or more nucleoli, in their cytoplasm small, dense mitochondria, sporadically small primary lysosomes and a poorly developed Golgi apparatus were observed (Fig. 3).

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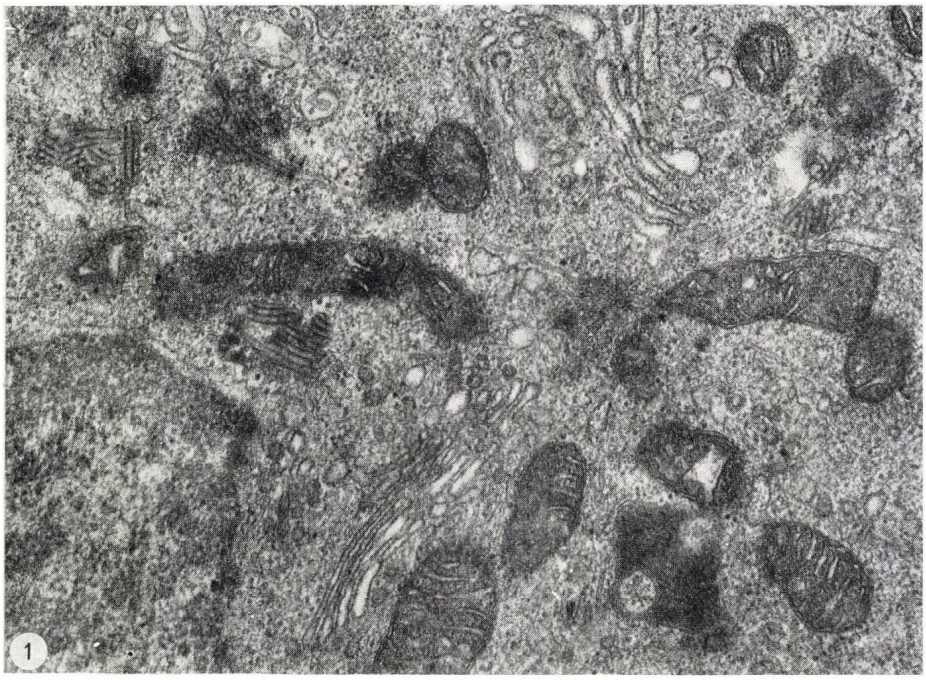
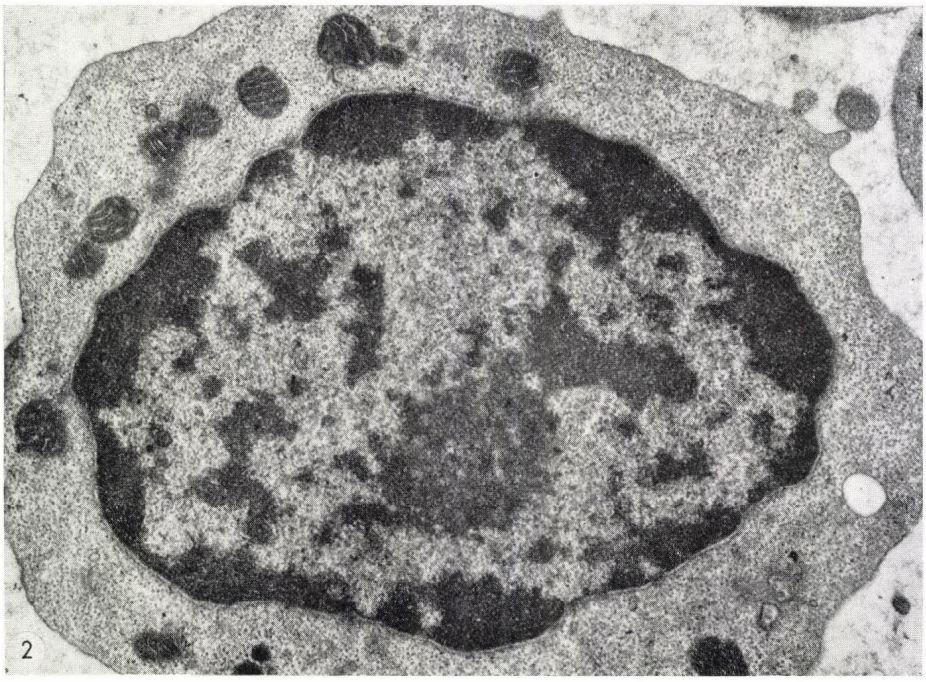
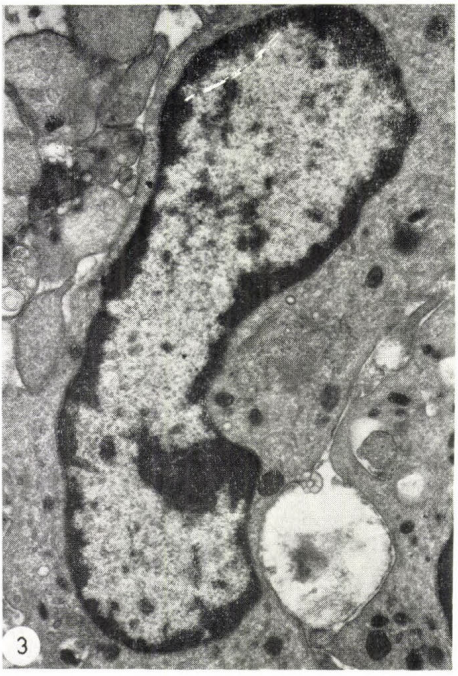
Fig. 1. Part of a large lymphocyte from the peripheral human blood. The large cytoplasm contains well-developed Golgi apparatus, many large mitochondria, tubular structures and short, rough surfaced endoplasmic reticulum lamellae. ×37,400

Fig. 2. Medium-size lymphocyte obtained from peripheral human blood. In the spherical nucleus considerable nucleolus is present and small, dense mitochondria, pinocytotic vesicles, few lysosomes and free ribosomes are characteristic for the cytoplasm. ×19,500







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These were the cell types found in the original material. Now we describe the cells characteristic for each period of cultivation.

In the 24 and 48 h cultures many macrophages and perishing neutrophilic granulocytes could be observed. In both of them phagocytized cell debris and thrombocytes were seen. Lymphocytes containing autophagic vacuoles and phagosomes were also characteristic. The number of the latter decreased in later periods of the cultivation. They assumedly either had perished or had been transformed into macrophage-type cells (Fig. 4).

Two non-lymphoid cell types were present in 72 and 120 h cultures:

(1) Typical macrophages with numerous phagosomes (Fig. 5).
(2) Cells revealing macrophage and monocyte characters.

The latter could not be sharply distinguished from each other, and could not be ranged in either well-defined leukocyte type on the basis of their fine structure. Three variants were observed: (*a*) macrophage-type cells, (*b*) macrophage-like cells with large, dense lysosomes (Figs 6, 7) and (*c*) larger cells as compared with the former, revealing monocytic and epithelial characteristics and containing many small lysosomes (Fig. 8). Under the culture circumstances mentioned, accumulation of lipid droplets in the periphery of the cytoplasm was characteristic for all the four cell types.

As to the lymphocytes, the following was observed in 120 h cultures:

(1) Small lymphocytes with nuclei containing much heterochromatin and a small nucleolus (Fig. 9).
(2) Small lymphocytes called 'uropodic' or 'hand mirror' form (Fig. 10).
(3) Medium-size lymphocytes containing large, dense mitochondria and cytosome (Fig. 11).
(4) Cells revealing immunoblast characteristics, identified by their relatively young nuclei and narrow cytoplasm poor in organelles. These are considered as transitional forms towards typical immunoblasts (Figs 12, 13).

In the 72 h cultures eosinophilic and basophilic granulocytes still occurred, but no plasmocytic lymphocytes and 'PHA-blast' cells were seen.

In conclusion, most sensitive for the cultivation conditions are the neutrophilic granulocytes, but some types of lymphocytes degenerate soon as

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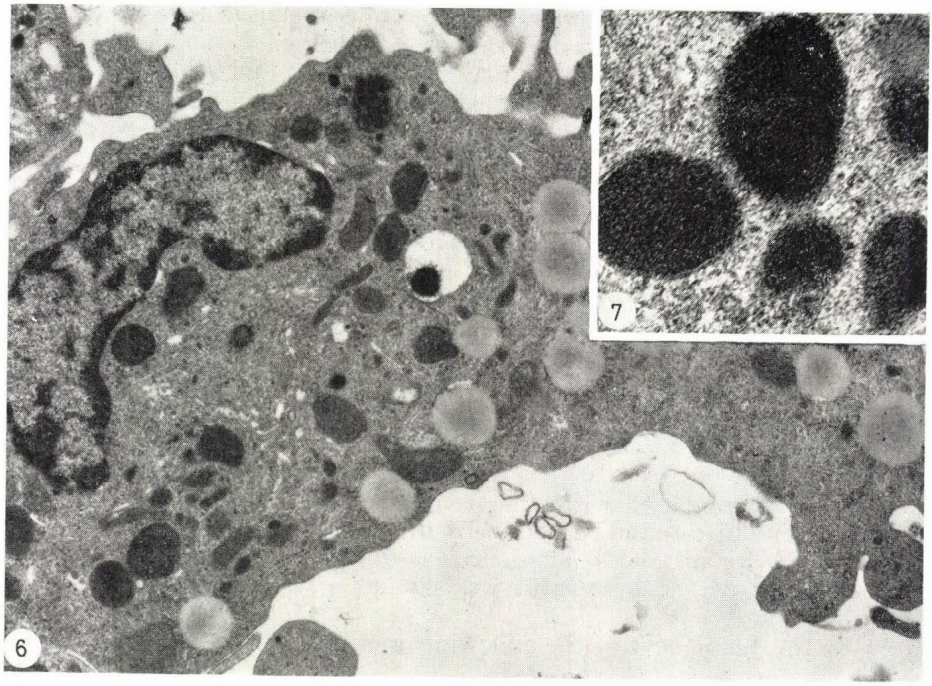
Fig. 3. This field demonstrates a typical peripheral monocyte. In the kidney-shaped nucleus small nucleoli, in the cytoplasm small, dense mitochondria, small primary lysosomes and an undeveloped Golgi apparatus are present. × 9,600

Fig. 4. This cell is a transitional form between lymphocyte and macrophage from a 24 h culture. The round nucleus with a small nucleolus is characteristic of a lymphocyte, the cytoplasm with the prominent phagosomes of macrophages × 13,000

Fig. 5. A typical macrophage from the 24 h culture. Note the different auto- and heterophagosomes. × 20,000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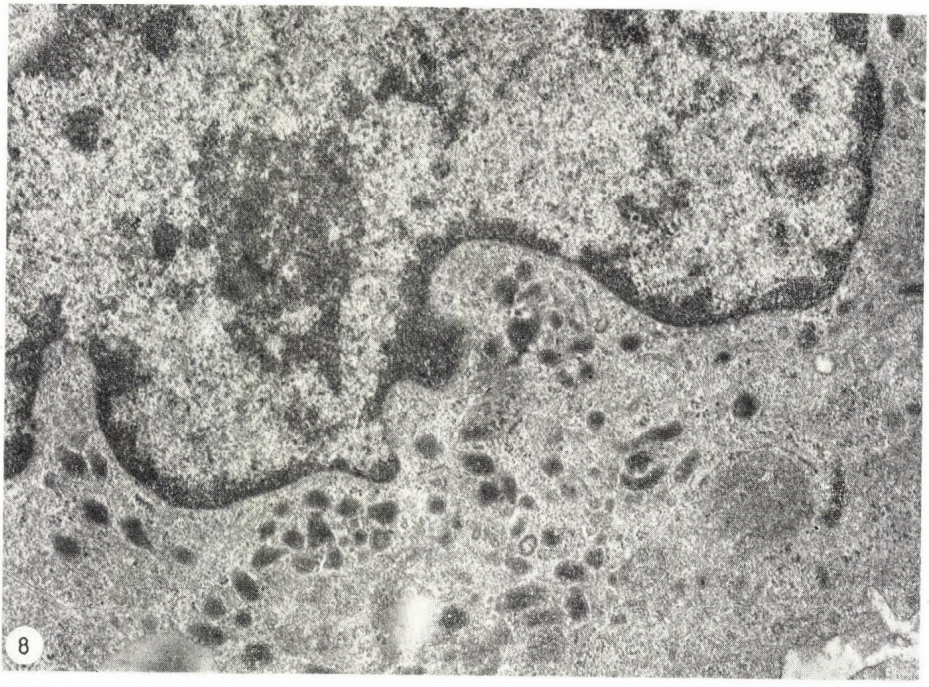
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well. It is not inconceivable that the more sensitive, sooner disintegrating lymphocytes correspond to short-life, those alive on the 5th day of cultivation, on the other hand, to long-life lymphocyte types. Further experiments are needed to decide whether this suggestion holds true. The cell types resistant to the cultivation conditions, not subject to any morphological alteration during cultivation are the small and, above all, the medium-size lymphocytes.

The phenomenon of hetero- and autophagy can be considered adaptation of the cells to the environmental factors. Cell debris, like other foreign substances, act as a macrophage transformation inducing factor. Monocytes, and in our assumption some lymphocytes, are transformed into macrophages under the influence of cell debris. In addition to macrophages, new cell types as macrophage-like, monocytoid and epitheloid cells, transitional forms and in long-term cultures fibroblast-like cells, multinucleated giant cells or lymphoblastoid cells can be formed of them.

In our cultures lymphoblasts arisen from spontaneous transformation, characteristic for cultures stimulated with PHA or other mitogens, could not been observed (Forteza-Vila et al., 1969; Balázs et al., 1970). The lymphocytes of younger character found can only be considered transitional forms towards 'PHA-blast' cells and immunoblasts (Balázs et al., 1970).

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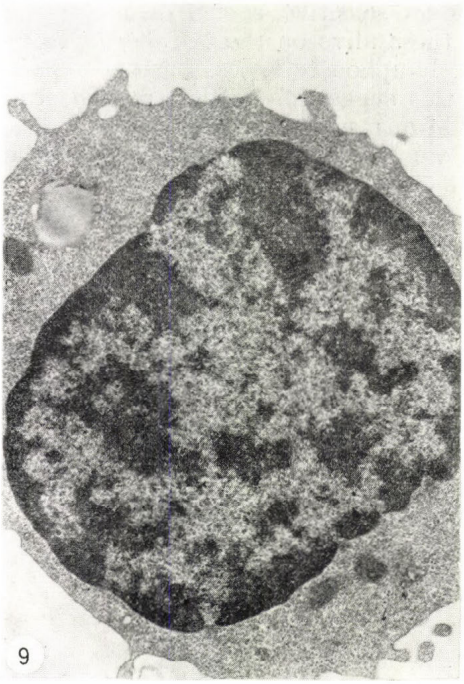
Fig. 6. Macrophage-like cell with large, dense lysosomes and lipid droplets from the 72 h culture. Centriolum and Golgi apparatus are also present. ×12,000

Fig. 7. Lysosomes from a macrophage-like cell. The granules are membrane-limited and the matrix has a finely granular substructure. ×32,300

Fig. 8. A young, large cell revealing monocytoid and epithelial characteristics, from the 72 h culture. A large nucleolus is present in the nucleus, and the cytoplasm contains many primary lysosomes, sporadically microfilaments and polyribosomes. ×19,500



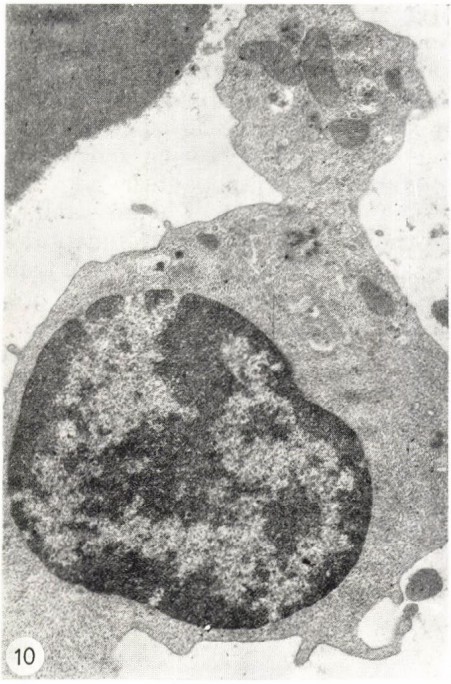


Fig. 9. Small lymphocyte with a small nucleolus and few cytoplasmic organelles $\times 13{,}600$

Fig. 10. A 'hand mirror'-form medium-size lymphocyte. This shape is characteristic for the sliding lymphocytes. $\times 11{,}000$

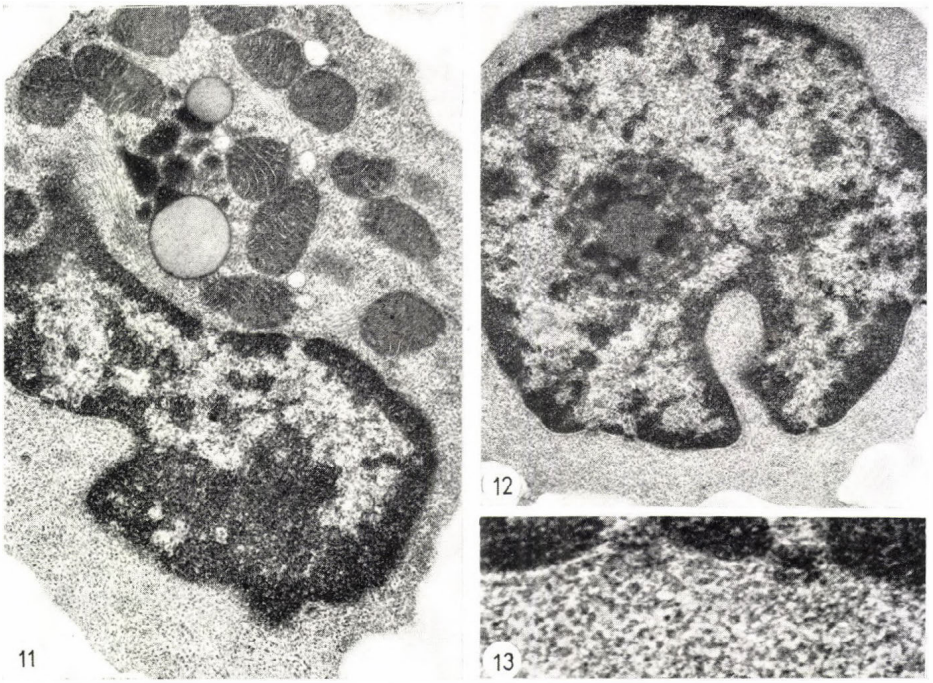


Fig. 11. Medium-size lymphocyte containing a nucleolus and many cytoplasmic organelles. The large, dense mitochondria, cytosome and filaments are characteristic for this cell type. $\times$ 17,000

Fig. 12. Lymphocyte revealing transitional immunoblast characteristics. Note the prominent nucleolus, and the lack of cytoplasmic organelles. $\times$ 13,400

Fig. 13. Nuclear pores, few polyribosomes and many free ribosomes are also characteristic for this lymphocyte type. $\times$ 61,800

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DIE ULTRASTRUKTURELLE UNTERSUCHUNG VON ZELLEN NACH IHRER LEBENDBEOBACHTUNG IN DER KULTUR*

von

W. O. GROSS**

HISTOLOGISCH-EMBRYOLOGISCHES INSTITUT DER UNIVERSITÄT LAUSANNE, SCHWEIZ

Es gibt für den Gewebezüchter nichts Reizvolleres, als im Elektronenmikroskop eine Zelle zu betrachten, die er vorher in der Kultur hat leben sehen. Ultrastrukturelle Differenzen zwischen normalen und malignen Zellen (Porter und Thompson, 1947; Gey und Bang, 1951), die Kultivierung von Neuroblasten (Barski et al., 1949), die Zilienstruktur von Fungen und Algen (Manton et al., 1952), die Identifizierung des endoplasmatischen Reticulums mit dem Ergastoplasma (Porter und Thompson, 1953), Bakteriophageneffekte (Murray und Wyckoff, 1953), die Aktivität verschiedener Zellorganellen in der Kultur (Gey et al., 1954), Mitosestudien in HeLa-Zellen (Robbins und Gonatas, 1964), Chromosomenstrukturen von Speicheldrüsenzellen (Gay, 1955), Strukturstudien an enukleierten Amnionzellen (Micou et al., 1962), Mitosevorgänge in Tradescantia (Sparvoli et al., 1965), Entwicklung von Rückenmarkskulturen (Bunge et al., 1965), Leukocyten in verschiedenen Phagozytosestadien (Egerberg, 1965), die Feinstruktur von Sexchromatinkörpern (Wolstenholme, 1965), Effekte von Laserstrahlen auf KB-Zellen (Storb et al., 1966; Lesourd und Chevance, 1967), Meiosestadien von HEp-Zellen (Price, 1967), Konfiguration von Haftstellen der Chang-Leberzellen an Millipore-Filtern (Dalen und Nevalainen, 1968), die Entwicklung von Zahnanlagen in der Kultur (Kumegawa et al., 1968), Verhältnis von Lewis'schen Pinocytosevakuolen zu Mikropinocytosebläschen (Gross und Riedel, 1969), Natur der Asphaltflecke von isolierten Herzmuskelzellen (Gross und Müller, 1971), das Zell-Virusverhältnis in Fibroblastenkulturen (McCombs et al., 1968), Kinematographische Zellforschung (Bereiter-Hahn und Egner, 1968), Radioautographische Untersuchungen von Zellteilen (Möller und Cirelli, 1970), Reizleitungsphänomene (DeHaan et al., 1969/1970), das Verhalten von Keratocyten und Melanocyten des Meerschweinchens in der Kultur (Branson, 1971), die Differenzierung von glatten Muskelzellen in der Kultur (Campbell et al., 1971) — und andere interessante Forschungsvorhaben haben die Experimentatoren zu Verfahren inspiriert, der Zelle vom mikroskopischen Bild der Kultur zum Elektronenmikroskop eine Brücke zu schlagen. Es fällt aber auf, daß man in den jüngsten Jahren für die Elektronenmikroskopie von kultivierten Zellen viel häufiger erst von der fixierten oder noch öfter sogar von der schon eingebetteten Kultur ausgeht, als daß man die Zelle, deren EM-Bild man anfertigt, wirklich im lebenden Zustand gesehen

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** Unter Mitarbeit von Fr. Ch. Müller.

hätte. Wohl ist eine Vorlage von fixierten oder eingebetteten Zellen zu manchem Studium der Ultrastruktur als Basis ausreichend. Denn die Fixierung mit Glutaraldehyd und Osmiumtetroxid, wie sie für die Elektronenmikroskopie benutzt wird, erhält einer Zelle viel ihres ursprünglichen Aussehens.

Sobald man jedoch die Einzelheiten, die das EM-Bild bringt, mit Funktionsabläufen in der Zelle in Verbindung bringen will, muß der elektronenmikroskopischen Aufbereitung die Lebendbeobachtung der Zelle vorausgehen. Die Methodik, eine Zelle aus der Kultur unter das Elektronenmikroskop zu versetzen, ist von ihrem Beginn ab, wo Zellteile in ihrem Ganzen von den Elektronenstrahlen zur Bildererzeugung durchdrungen werden mußten (Porter et al., 1945), jetzt seit man nach Borysko und Sapranauskas (1954) Schnitte von Kulturen anfertigt und man seit Bloom (1960) die in der Kultur gesehene oder behandelte Zelle im EM wiederfindet, durch die sich weiter verbessernden Indentifizierungsverfahren so sicher geworden und liefert durch die sich in jenen Jahren gleichzeitig weiterentwickelnden Fixierungs-, Einbettungs- und Schneidetechniken so einwandfreie Bilder, daß ein Gewebezüchter jetzt ohne weiteres die elektronenmikroskopische Untersuchung der Lebendbeobachtung seiner Zelle anschließen kann.

Lassen Sie mich dafür ein Beispiel bringen: Hier wird ein Vorgang in der lebenden Zelle mit Zeitraffer dargestellt. Eine isolierte Herzmuskelzelle des Hühnerembryos zeigt neben den uns bekannten Organellen wie Kerne mit Nucleolus, Fetttröpfchen und Mitochondrien, paraplasmatische dunkel getönte Einschlüsse. Diese als Asphaltflecke benannten Gebilde ändern sich. Was jetzt vor Ihnen abläuft, ist während $1^1/_2$ Stunden geschehen. Fettkügelchen und Mitochondrien bewegen sich schnell. Die Flecke bleiben an ihrem Ort, ändern aber langsam ihre Konturen. Was ich Sie besonders zu beachten bitte, ist diese Stelle im Plasma. Es bildet sich hier ein neuer Fleck, der Punkt wird stetig größer; schließlich verbindet er sich mit dem schon vorhandenen größeren Fleck. Was ist hier geschehen? Die EM-Untersuchung soll Aufschluß geben.

Im linken Bild erscheint diese Zelle noch einmal im Phasenkontrastbild (Abb. 1), ihm entspricht das noch schwach vergrößerte EM-Bild des rechten Projektors (Abb. 2). Die Identität der äußeren Form beider Zellbilder ist unverkennbar. Bitte auch die gleiche Form des Nucleolus zu beachten. Auch die Mitochondrien-Anhäufungen hier und dort entsprechen sich. Außerdem können wir Fetttröpfchen auf beiden Bildern identifizieren. Nun interessiert uns am meisten die Stelle, an der der Fleck entstanden ist. Das war hier an dieser Schwärzung im EM-Übersichtsbild. Um was es sich dabei dreht, sollen die höheren Vergrößerungen zeigen. In diesem Bild (Abb. 3), wo gerade noch der Kern mitgeschnitten ist, liegt der Fleckenbezirk am rechten Rand. Im nächsten Bild, das zur rechten Zellgrenze reicht (Abb. 4), ist er am linken Rand, und hier im dritten Bild (Abb. 5) haben wir ihn im vollen Visier. Bei der stärksten Vergrößerung löst er sich auf in Partikel, die einen ungefähren Durchmesser von 180–300 Å haben. Die Abb. 6 zeigt bei gleicher Vergrößerung den Fleckenbezirk in einer anderen Schnittebene.

Das ist die Natur dieser Einschlüsse generell. In seiner Fortsetzung zeigt der Film einen pulsierenden Komplex von Herzmuskelzellen. Gepaart

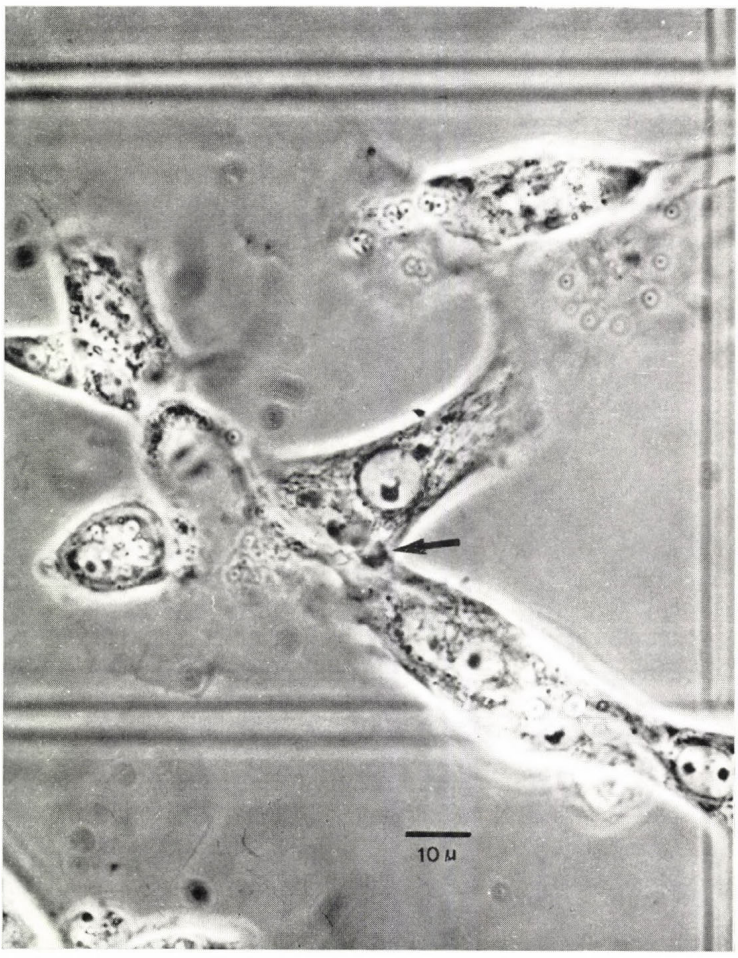


Abb. 1. Phasenkontrastbild einer isolierten Herzmuskelzelle in der Kultur. Innerhalb $1^1/_2$stündiger Beobachtung war der mit dem Pfeil bezeichnete Fleckenteil aus einem punktförmigen Anfang neu entstanden

mit Mitochondrien, Fetttröpfchen und Pinocytosevakuolen sehen wir auch wieder Asphaltflecke. Der Vergleich des EM-Übersichtsbildes mit dem Bild der lebenden Zelle erbringt die identischen Organe. Fetttröpfchen der Nummern 1–4, Pinocytosevakuolen der Nummern 1–5, Mitochondrien der Nummern 1–3. Auch hier lösen die stärkeren Vergrößerungen die Flecke zu Gebilden von 180–300 Å Durchmesser auf.

Schließlich bringt der Film noch einen kleinen pulsierenden Komplex mit sehr starkgetönten Asphaltflecken. Dieser Zipfel des EM-Fotos entspricht jener Stelle des Phasenkontrastbildes, und die höhere Auflösung bringt in dem vollen Fleck wieder die Körperchen, deren Durchmesser den β-Teilchen des Glykogens entsprechen. Sie liegen so lose, daß die histochemischen Untersuchungen lange Zeit wegen der Fixierungsschwierig-

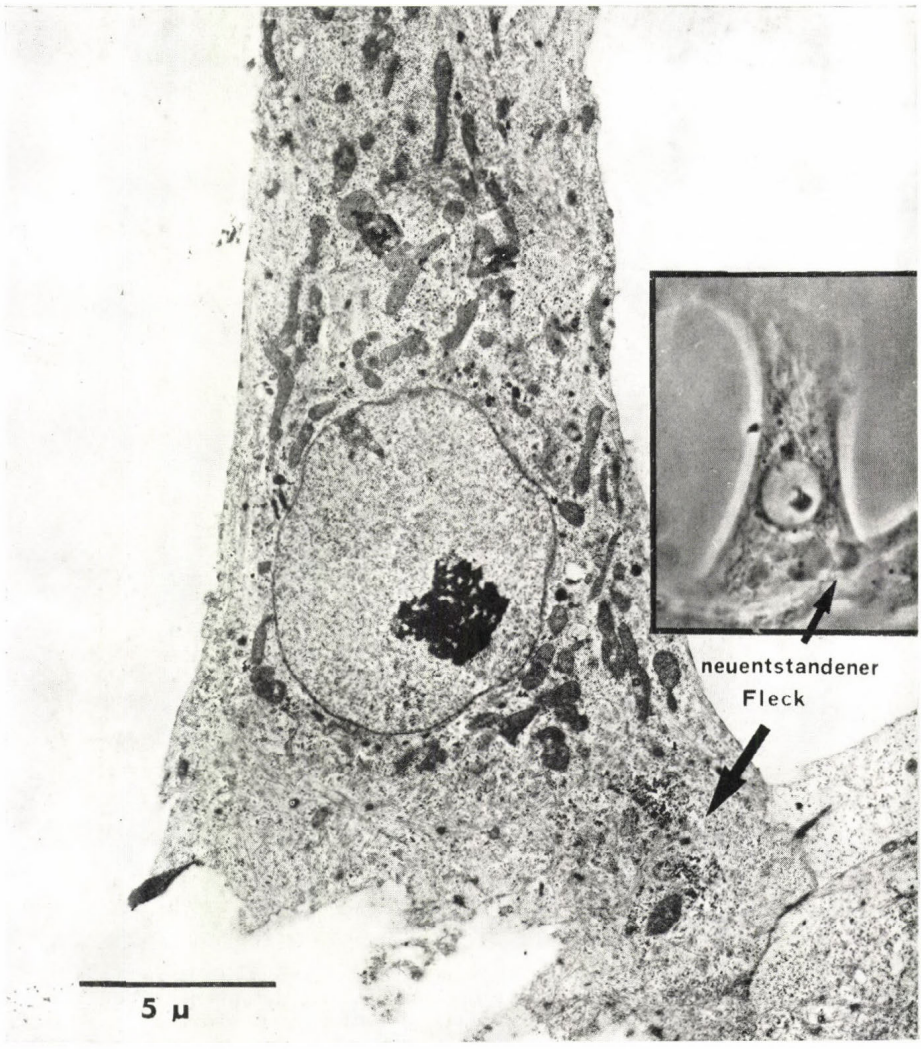


Abb. 2. EM-Übersichtsbild der gleichen Zelle. Der Pfeil zeigt auf die Stelle, wo der Fleck entstanden ist. Sie ist durch eine Schwärzung charakterisiert

keiten scheiterten. Es gelangen dann aber doch eine Reihe von Färbungen. Zunächst nach Gendrefixierung eine Fermentprobe. Hier mit Wasser und hier mit Diastase. Man sieht, daß das, was nach dem Fixieren übrig blieb, nach der Fermenteinwirkung weggelöst ist. Für diese schlagende Zelle haben wir im weiteren Filmstreifen positive histochemische Reaktionen erhalten: Joddampf (Phasenbild, durchscheinendes Bild), Karminfärbung (Phasenbild, durchscheinendes Bild) und schließlich PAS-Färbung (Phasenbild und durchscheinendes Bild).

Was man also in diesen elektronendichten Stellen gesehen hat, war Glykogen. Es sind locker liegende β-Teilchen nach Drochmans (1962)

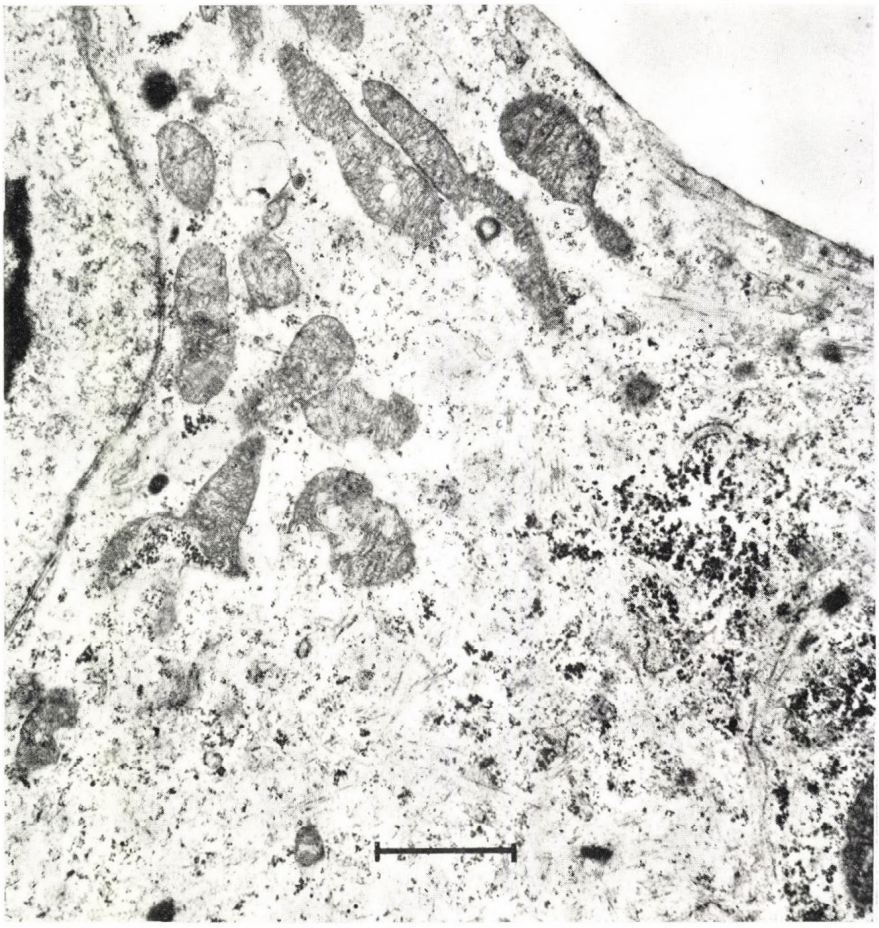


Abb. 3. Ein Teil der gleichen Zelle, der vom Kern bis in den Fleckenbezirk reicht

Terminologie. Und was wir im Film gesehen haben, war die mit Zeitraffer zur Erscheinung gebrachte Glykogensynthese in der lebenden Herzmuskelzelle des Hühnerembryos. Den neugebildeten Fleck im EM-Bild wiederzugeben, war für diese Behauptung wichtig, weil noch weithin unbekannt ist, daß man Glykogen im Phasenkontrastmikroskop sehen kann und deshalb ein Beweis durch Parallelschlüsse nicht überzeugt hätte.

Was jetzt die derzeitige Technik anlangt, die Zelle von der Beobachtung der Kultur mit dem Phasenkontrastmikroskop ab bis zum Schneiden mit dem Ultramikrotom nicht zu verlieren, kann man nach zwei Prinzipien verfahren. Bei dem ersten Prinzip beginnt man mit den Kunstgriffen erst, wenn man die Kultur unter dem Mikroskop hat.

Dieses bis in die letzten Jahre praktizierte Standardverfahren (z. B. Price, 1967) markiert mit einem Diamantschreiber zuerst die Rückseite der Stelle des Deckgläschens, wo auf der Kulturseite die beobachtete Zelle

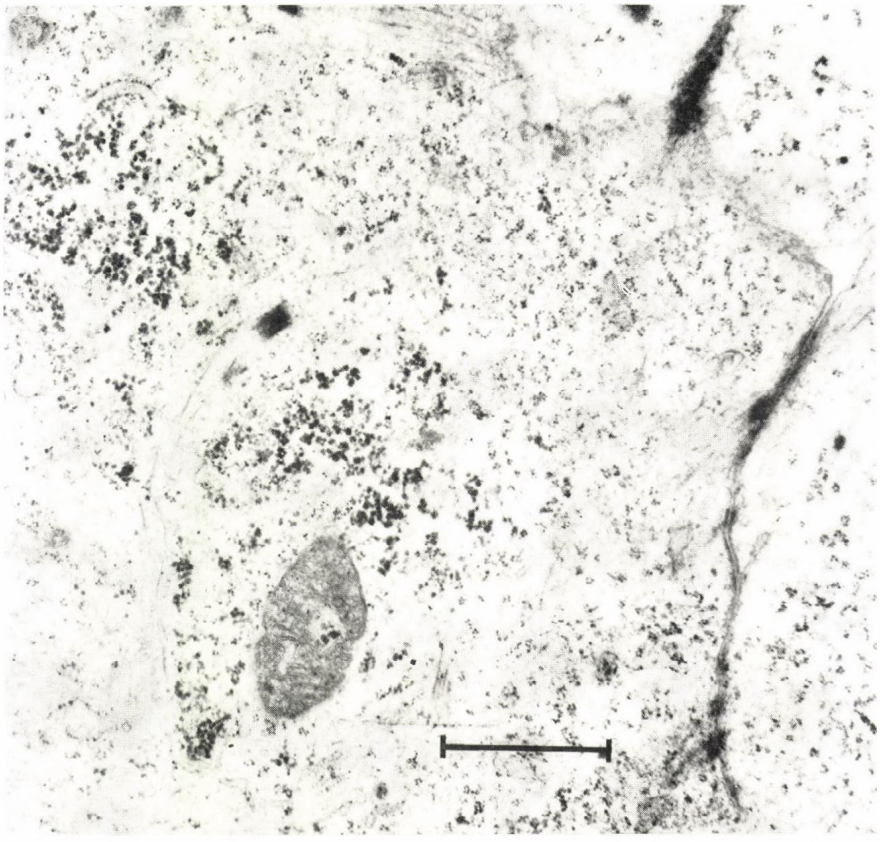


Abb. 4. Die von der Zellgrenze ausgehende Strecke zum Fleckenbezirk

liegt. Das ist der Anfang. Und nun entwickeln sich zwangsläufig darauf z. B. folgende Manipulationen:

(1) *Markieren des Kreises.* Der Kreis, den man vielleicht mit einem Durchmesser von 1 mm gezogen hat, wird nach Öffnen und Fixieren der Kultur mit einem Fettstift selbst noch markiert. Dazu muß man, wenn man zum Beobachten der Kultur und Anbringen des Kreises ein Umkehrmikroskop benutzt hat, die offene Kammer umkehren und Sorge dafür tragen, daß das Präparat trotzdem auf der Kulturseite feucht bleibt.

Gay sprach 1955 von einer Markierung ihrer Quetschpräparate zur späteren Einbettung. Über die Art und Weise fehlen bei der Autorin nähere Angaben. Bloom hat 1960 einen Kreis mit dem Diamantstift auf die Rückseite des Deckgläschens gesetzt. Er fand damit die Zelle nach Färbung in seiner Zentriervorrichtung und konnte sie damit auf das Visierkreuz ausrichten, bevor er einbettete – eine Aufsuchmethode, die Silvestre et al. im Umkehrmikroskop 1961 wiederholten. Bereiter-Hahn und Egner (1968) begannen auch ihre Identifizierungstechnik von lebenden Zellen mit dem Außenkreis, außerdem Wolstenholme (1965) sowie Price (1967).

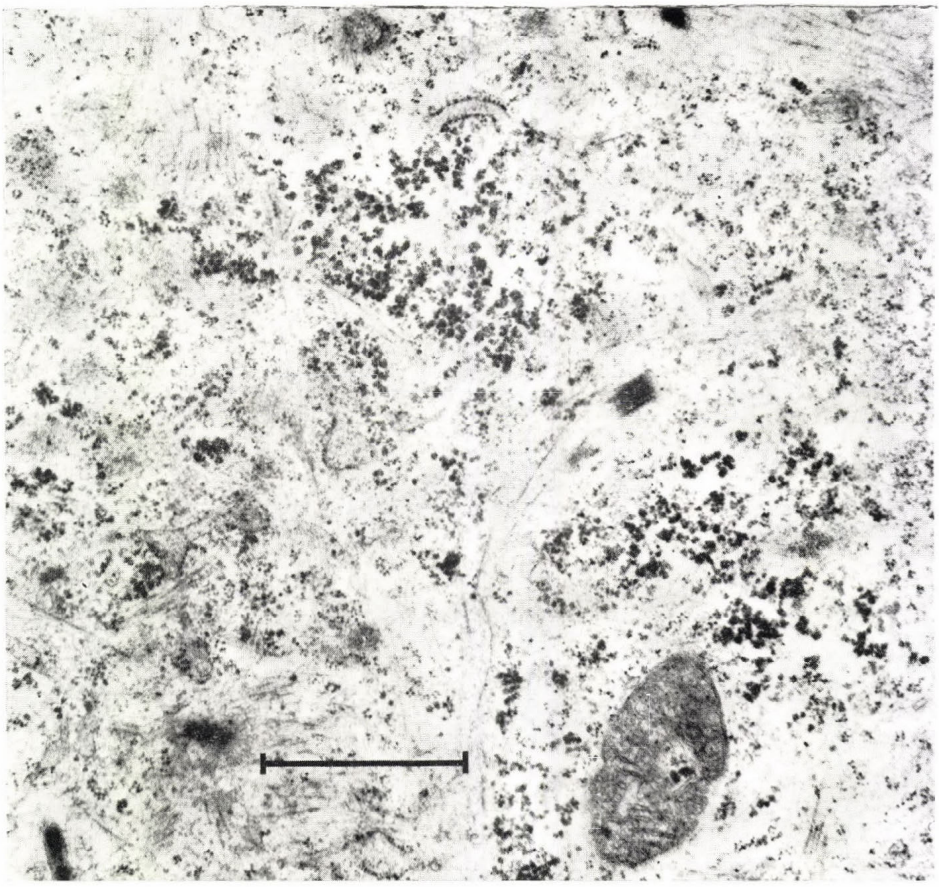


Abb. 5. Der eigentliche Fleckenbezirk. Bei dieser Vergrößerung läßt sich der Durchmesser der Einzelkugeln mit 180–300 Å bestimmen. Er ist mit dem der β-Teilchen des Glykogens gleich

(2) *Kreise auf der Kulturseite.* An Hand dieser Markierung findet man im Vertikalmikroskop den Kreis auf der Außenseite des Deckgläschens und nach Fokusierung der Kulturseite auch die ausgewählte Zelle. Hier erhält man allerdings ein gegenüber der ersten Beobachtung umgekehrtes Bild. Statt des Objektivs setzt man nun den Diamantschreiber ein und ritzt damit um die ausgesuchte Zelle den zweiten Kreis mit etwa halb so großem Durchmesser. 0,5 mm Durchmesser eines Kreises erfaßt natürlich mehrere Zellen; deshalb ist es von Vorteil, wenn man die ausgewählte möglichst in die Mitte des Kreises gelegt hat. Die Gefahr, die Zelle durch den Diamantstift zu verletzen, verhindert, kleinere Kreise zu benutzen.

Robbins und Gonatas (1964) verzichteten auf den äußeren Kreis. Sie machten einen Kreis in die Kultur. Das war aber nur möglich, weil sie die Zellen vorher fixiert hatten. Der Kreis auf der Innenschicht des Deckgläschens zeichnete sich auf dem Araldit-block später ab. Robbins und Gonatas führten den Abdruck auf die angeritzte Kohleschicht zurück, die sie zum Zwecke der späteren Ablösung des Einbettungsmittels

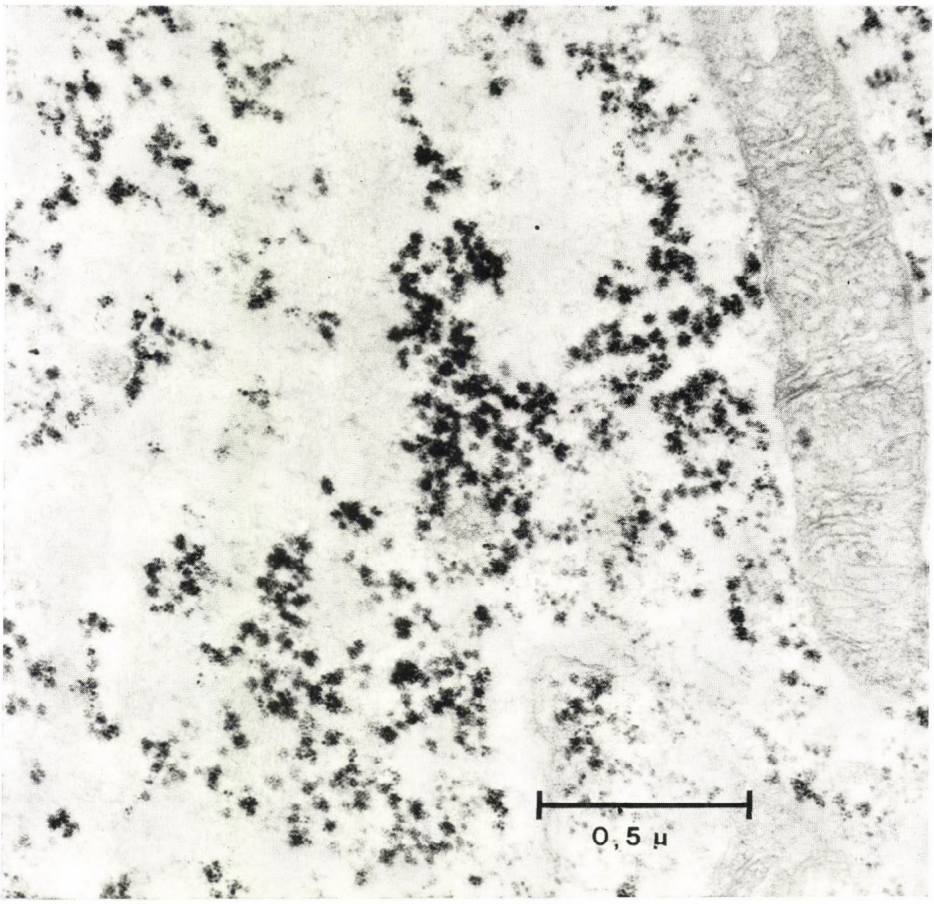


Abb. 6. Aus einem hier nicht gezeigten Übersichtsbild einer anderen Schnittebene ließ sich am Ort des Flecks diese Vergrößerung herstellen. Das Glykogen ist wieder erkennbar

(Araldit) aufgedampft hatten. Auch Montgomery et al. (1966) sowie Price (1967) nehmen den Abdruck als einen Effekt der Verletzung der Kohleschicht. Egeberg (1965) benutzte ebenfalls den Diamanten. Er bezeichnete aber damit die schon in Epon eingebetteten Zellen. Ebenso verfuhren Brinkley et al. (1967) und Zagury et al. (1968).

Storb et al. (1966) präparierten Deckgläschen der Rose-Kulturkammer auf der späteren Kulturseite mit 3 Kreisen von 200–400 μm. Damit dort die Kapseln aufgesetzt wurden, waren auf der Rückseite des Deckglases ebenfalls Kreise angebracht. Diese waren größer und nahmen die Innenkreise in ihren Bereich auf. Erst die eingeritzten Deckgläser wurden mit Formvar überzogen und hierauf die Zellen zur Kultur gesetzt. Das Formvar war mit einem Eponring aufgeklebt. Die eingravierten Kreise haben sich dann trotz des darüber befindlichen Formvar auf den Eponblock abgedruckt. Das beweist, daß in den Verfahren von Robbins und Gonatas es nicht die Kohle war und ihre Verletzung, die den Abdruck gab, sondern die Diamantspur im Glas.

(3) *Färben der Zelle mit einer Alkohol-unlöslichen Farbe.* Nachdem die Zelle mit einem Kreis bezeichnet ist, kommt sie zum Trimmen des Eponblocks besser heraus und läßt sich eher schonen, wenn sie gefärbt ist. Der Kreis ist ja nur ein Anhaltspunkt für eine genaue Lokalisierung.

Das Anfärben der Zellen so, daß man ihre Formen im Einbettungsmittel später wiedererkannte, war schon ein Bestandteil der Bloomschen Identifizierungstechnik (1960). Aus seiner Beschreibung geht nicht sicher hervor, ob bei ihm die elektronenmikroskopische Anfärbung mit Osmiumtetroxid z. B. schon ausgereicht hat oder ob die Kultur auch noch mit einer anderen Farbe imprägniert war. Storb et al. (1966) benutzten Janusgrün oder Nilbau, Cresylblau oder Methylenblau, um die Zellen im Block stärker hervortreten zu lassen. Auch Gorycki (1966) färbte die Zellen mit Methylenblau an. Price färbte mit Anilinblau nach der Fixierung in Glutaraldehyd + OsO_4, Dalen und Nevalainen (1968) mit Hämatoxylin.

(4) *Zentrieren der Kapsel über der Zelle.* Dazu wird eine Beem- oder Gelatinekapsel gekappt und in eine Zentriereinrichtung eingesetzt, die in einen Spezial-Deckglashalter paßt. Mit dem Licht durch die Kapsel in einem Umkehrmikroskop wird die Zelle aufgesucht und in die Mitte des aufsitzenden Kapselrandes gebracht. Darauf wird die Kapsel gefüllt und samt Zentriereinrichtung in den Heizofen bei 60 °C gestellt.

Der erste Apparat, mit dem man die ausgewählte Zelle in den Mittelpunkt des Blockes richten konnte, stammt von Bloom (1960), wiewohl Nebel und Minick (1956) und auch Nishiura und Rangan (1960) Halter für das Aufsetzen von Kapseln konstruiert hatten — Vorrichtungen, die später dadurch überflüssig geworden sind, weil das flüssige Methacrylat von einem z. T. polymerisierten Methacrylat oder dem noch zäheren Epon ersetzt wurde und der Inhalt einer aufs Deckglas aufgesetzten Kapsel ohnehin nicht auslief. Die Vorrichtung von Bloom bestand im Prinzip darin, die Zelle mit einem Kreuz in Deckung zu bringen, das sich auf einem Glasplättchen eingeritzt, im genauen Zentrum eines Metallringes befand. In diesen Ring paßte ein zweiter, durch den ein durchscheinender Stab gesteckt war, dessen Spitze mit der eingebetteten Zelle verklebt wurde. Silvestre und Mitarb. benutzten eine ähnliche Vorrichtung 1961. Obgleich Robbins und Gonatas (1964) durch den auf die Schichtseite gelegte Kreis die Zentriervorrichtung glaubten entbehrlich gemacht zu haben und Storb et al. (1966) die Kreise in das vorzubereitende Deckgläschen auf die Kulturseite setzten, haben Price (1967) und auch Bereiter-Hahn und Egner (1968) noch Zentriereinrichtungen erfunden, wovon die praktischste von den zuletzt genannten Autoren stammt.

Man kann die Spezialvorrichtungen jedoch umgehen, wenn man, wie Wolstenholme (1965), die Kultur nur in einer Schicht und nicht mit einer ganzen Kapsel einbettet. Diese kleine Platte wird auf einen Objektträger unter ein Mikroskop gebracht. An Hand des Außendiamantkreises des noch darauf befindlichen Deckgläschens können die Zellen nochmals eingestellt und mit dem Nonius des Kreuztisches örtlich festgelegt werden. Nach den Noniuszahlen sind sie wieder auffindbar, wenn inzwischen das Deckglas z. B. mit CO_2-Eis abgesprengt ist. Jetzt kann man die Zellen im Einbettungsmittel erneut z. B. mit einer Rasierklinge markieren. Die Umrandung von etwa 0,1 mm findet man beim Trimmen wieder, wenn aus der Platte des Einbettungsmittels inzwischen die Stückchen mit den Zellen auf einen Zylinder des Einbettungsmittels geklebt sind.

Nach einer Abbildung im russischen Virologischen Lehrbuch von Chdanow und Gaidamowitsch (1966) zu schließen, hat auch Bykowskij (1966) diese Methode benutzt. Eine Beschreibung ist leider nicht beigegeben. Die Schwäche dieses Verfahrens ist die Markierung der Zelle im Einbettungsmittel, da die Umrandung für das Trimmen umso vorteilhafter ist, je näher sie an der Zelle verläuft. Hier besteht jedoch die Gefahr der Verletzung oder gar des Verlustes der ausgewählten Zelle.

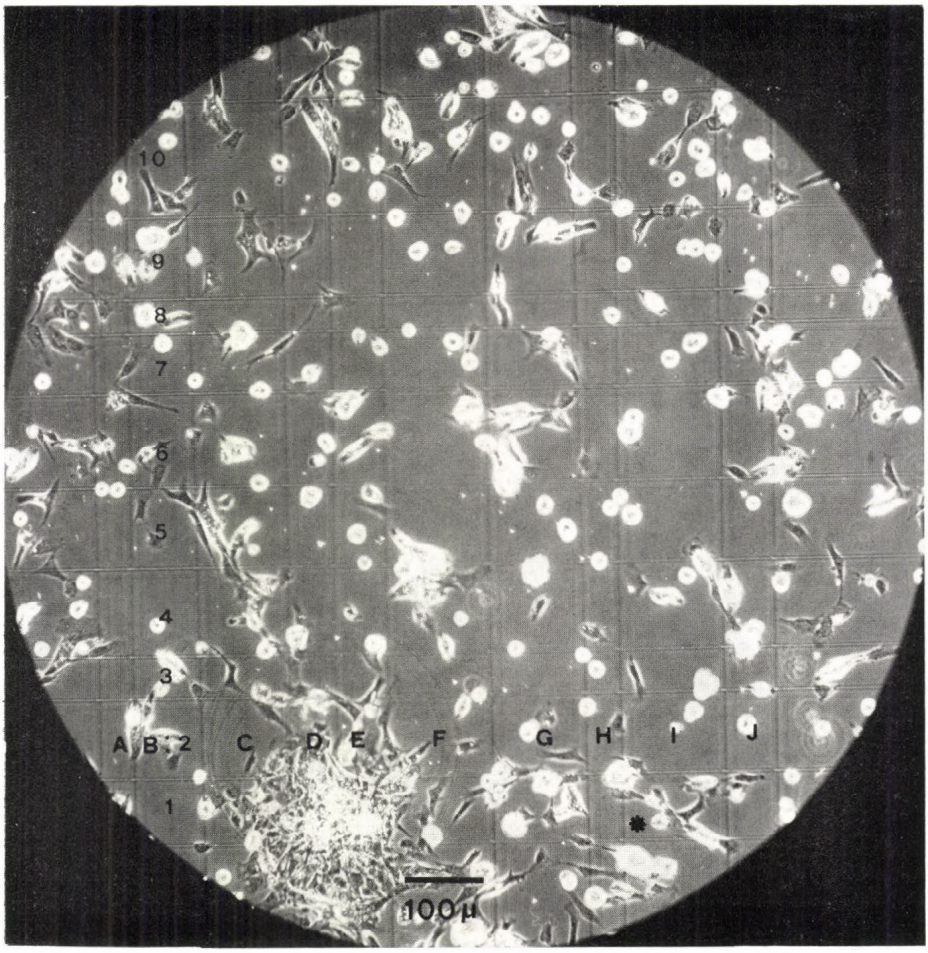
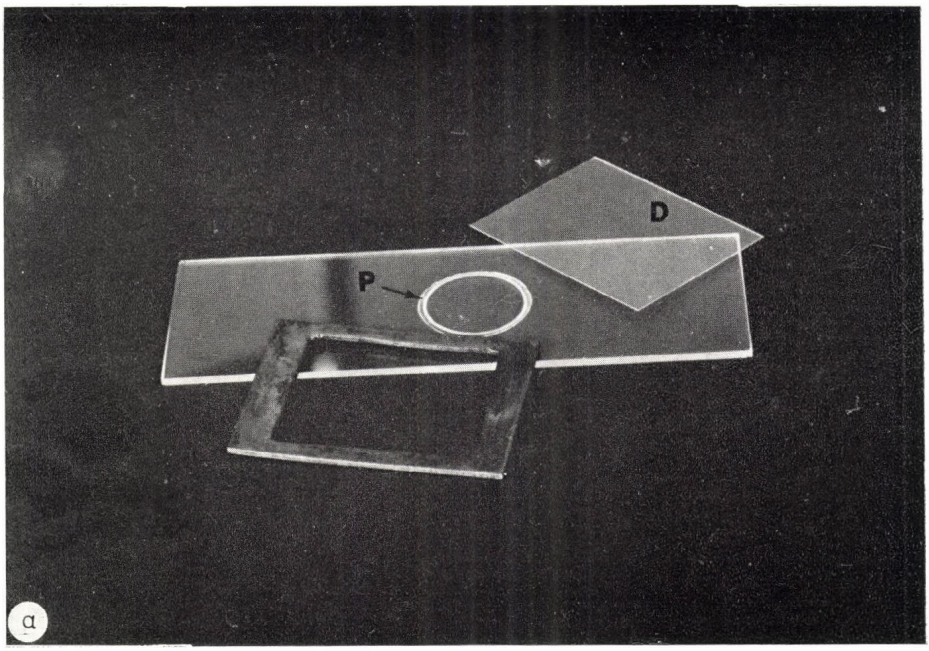


Abb. 7. Übersichtsbild der Kultur, aus der die Zelle der Abb. 1 für das Filmen der Fleckenentstehung ausgesucht worden war. Außer den Zellen werden die ins Deckgläschen eingravierten Striche für das Netz und den Kreis sichtbar. Die ausgewählte Zelle hatte sich im Quadrat I : 1 des Gitters abgesetzt (Sternchen)

Das Anbringen des äußeren Kreises, seine Markierung mit Farbstift unter dem Mikroskop, das Setzen eines zweiten Kreises auf die Kulturschicht, das Färben der Zelle, das Zentrieren der Zelle auf die Eponkapsel wird in dieser oder jener Form immer ausgeführt werden müssen, wenn mit den Manipulationen erst bei der fertigen Kultur begonnen wird. Alle 5 Kunstgriffe werden aber entbehrlich, wenn man sich beim Anlegen der Kultur die Lokalisierung der Zelle für das EM-Präparat schon vorbereitet hat. Das ist das zweite Prinzip.

Storb et al. hatten schon 1966 3 Kreise auf die Kulturschicht des Deckgläschens gesetzt. Diese Zeichen haben sich auf der Fläche des vom Deckglas abgelösten Eponblockes abgedrückt. Den Experimentatoren genügten 3 Plätze pro Präparat, denn welche Zellen sich zufällig hier absetzten, war belanglos, weil sie diese Zellen zur



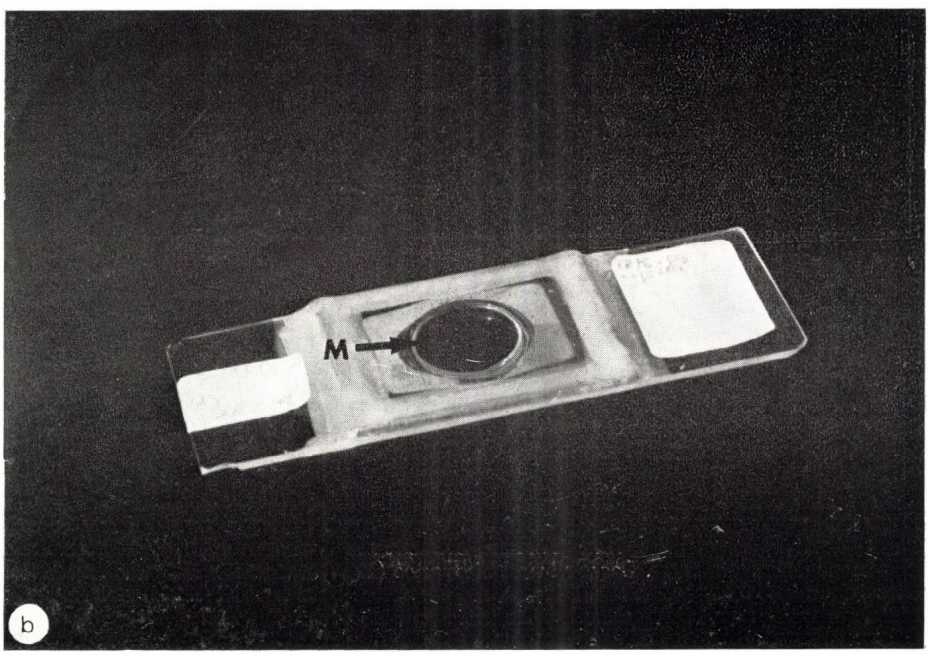


Abb. 8*a* und 8*b* zeigen die Bestandteile unserer Kulturkammer im losen und montierten Zustand. In das Deckgläschen (D) ist das bei diesem Foto unsichtbare Netz von 1,0 mm² Flächeninhalt eingeritzt. Der Paraffinring (P) hält das Medium (M) im Mittelpunkt der Kammer

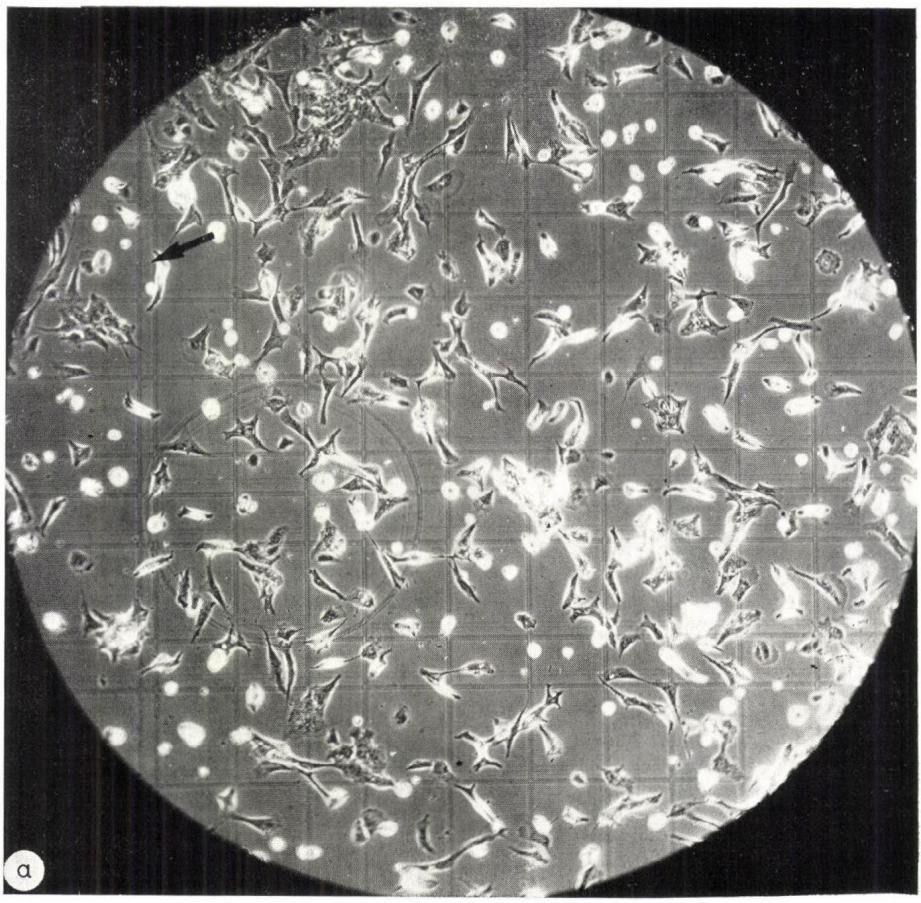


Abb. 9*a* und 9*b*. Kultur aus einem anderen Identifizierungsversuch. Die Zeichnung des Gitters ist sowohl bei Betrachtung der Kultur als auch beim Blick auf die Kapselfläche seitengleich. Die in den Quadraten liegenden fixierten Zellen der Abb. 9*b* entsprechen der der lebenden Kultur. Beide Netzbilder sind vom Netz der Abb 7 leicht zu unterscheiden: z. B. ist die A-Kolonne (Pfeile) bei den Abbildungen 9*a* und 9*b* enger als die übrigen, bei dem Gitter der Abb. 7 ist sie den übrigen Kolonnen eher gleich. In 9*b* erscheint beim Trimmen meist nur die Gravur. Im Foto werden die Zellen sichtbar, weil die Fläche in einer Scheibe von der Kapsel abgesägt und mit Immersionsöl auf einen Objektträger gesetzt worden war

Bestrahlung benutzten. Wenn Zellen unter mehreren anderen wegen ihrer eigenen Charakteristik ausgesucht werden sollen, braucht man an dem vorpräparierten Deckglas mehr definierte Orte. Lesourd und Chevance (1967) haben für freie Zellen ein EM-Gitterchen mit festwerdendem Plasma auf dem Deckglas befestigt. Die Zellen in den Maschen waren dreidimensional definiert. Da das Licht des Kondensors durch das Gitter dringen muß, ist dieses Verfahren für die Beobachtung einer auf dem Deckglas wachsenden Kultur unbrauchbar, auch wenn das Gitter ohne Plasma befestigt werden könnte. Dem von Storb et al. und Lesourd und Chevance angestrebten Prinzip, die örtliche Definition einer Zelle schon durch Ortsmarkierung der Kulturfläche zu erreichen, ist eine allgemeine Anwendbarkeit beschert, wenn man das Gitter von Lesourd und Chevance direkt in die die Kultur tragende Membran zeich-

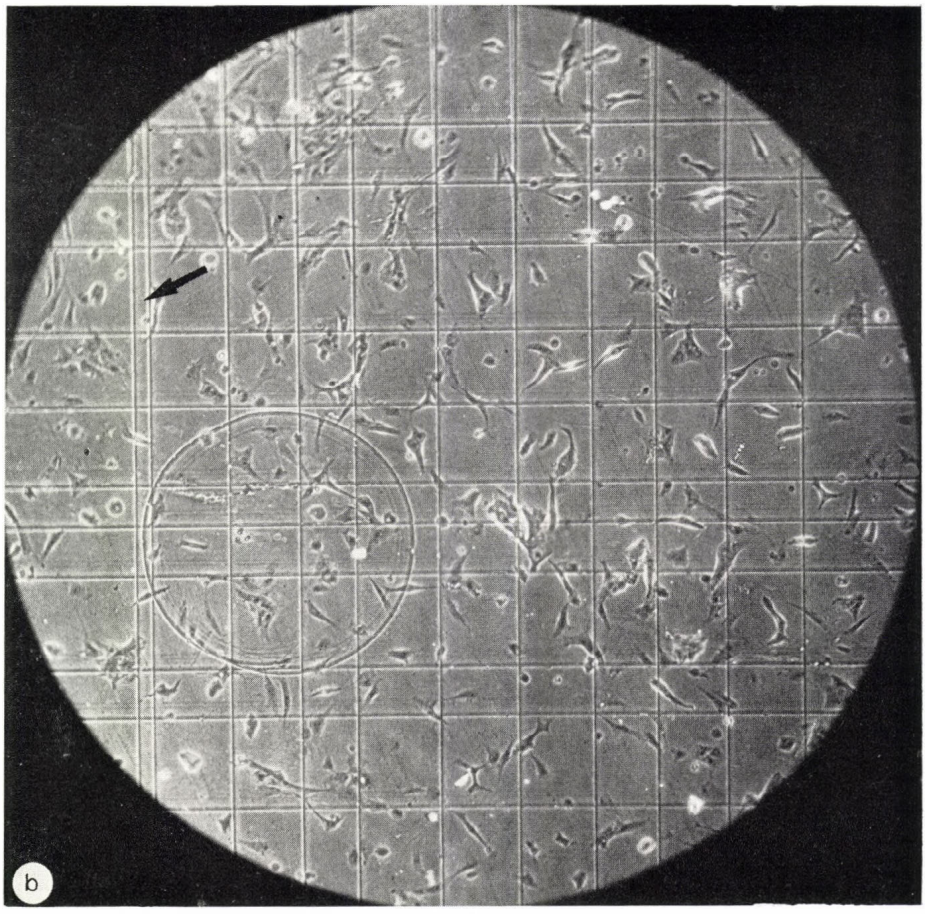


Abb. 9*b*

net, d. h. wenn man in die Kulturfläche des Deckglases statt der 3 Ringe von Storb et al. ein ganzes Netz von Planquadraten eingraviert.

Aus einem solchen Planquadrat haben Gross und Riedel (1969) die undulierende Membran einer stark pinocytierenden HeLa-Zelle unter das EM gebracht und in der Ultrastruktur die Massen der Mikropinocytosevesikel aufgedeckt, aus denen man sich die Bildung der Lewis'schen Pinocytosevakuolen zu denken hat. Auch die Ihnen demonstrierte Identifizierung ist so vor sich gegangen. Bei der Projektion der drei Zellen mit den Asphaltflecken konnten Sie Striche in dem Deckglas erkennen. Ich bringe noch einmal das Bild der Zelle, die den Fleck entstehen ließ (Abb. 1). Das Netz, in dem sie lag, ist im ganzen in diesem Übersichtsbild (Abb. 7) enthalten. Der Kreis links unten erlaubt die richtige Orientierung. Die Quadrate wurden mit A bis J nach der Seite und mit 1 bis 10 von unten nach oben bezeichnet. (Wie die ganze Kulturkammer, die dieses Deckgläschen enthielt, zusammengesetzt war, zeigen die Abbildungen 8*a* und 8*b*.)

Abb. 10. Das Eingravieren des Netzes. Das Deckgläschen liegt auf einem Objektträger. Sein Platz genau in der Mitte ist dadurch bestimmt, daß dem Objektträger als Schiene links und rechts zwei Lamellen (L) von Deckglasdicke aufgeklebt sind. Das Deckgläschen paßt gerade in ihren Zwischenraum. Der Diamantschreiber ist auf die Mitte des Deckgläschens gesetzt und ritzt unter Leitung der entsprechenden Ausschläge der beiden Noniuse (N_1 und N_2) die senkrechten und waagrechten Striche des Gitters ein

Dann ist die Zelle über alle Stadien der Präparation fürs EM hinaus bis zum Eponblock hin definiert. Sie liegt im Planquadrat I : 1. Man kann sich an Hand des Kreises orientieren, um das Planquadrat aufzufinden. Das Bild, das man beim Trimmen auf der Fläche des Blockes vom Netz sieht, ist mit dem bei der Betrachtung der Kultur seitengleich und nicht spiegelbildlich. Z. B. habe ich hier links ein Foto der Kultur (Abb. 9*a*), rechts eines mit Blick auf den Block (Abb. 9*b*). Man sieht in beiden Fällen den Kreis links unten. An der Abbildung sieht man auch die Unregelmäßigkeit der Netze.

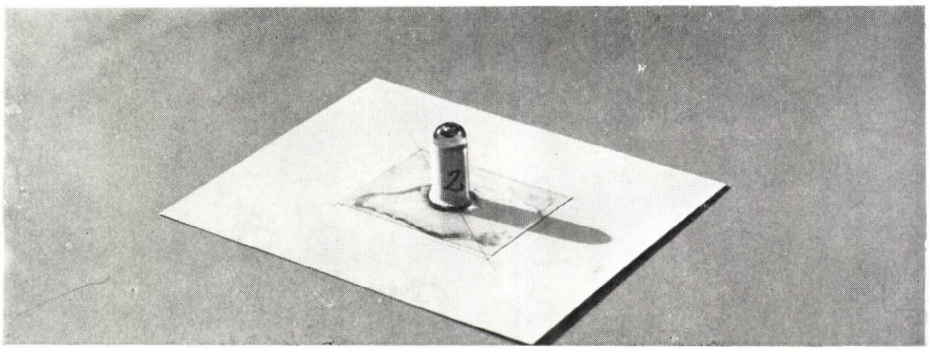


Abb. 11. Die auf die Mitte des Deckglases gesetzte Durcupankapsel trifft genau das Netz in der präparierten Kultur. Die Diagonalen in der Zeichnung des darunterliegenden Kartons leiten auf den richtigen Aufsetzpunkt

Wir haben bei den Abbildungen 9*a* und 9*b* links z. B. zwei eng aneinanderliegende Linien. Bei dem ersten Netz (Abb. 7) ist die A-Reihe nicht so eng. Dafür charakterisiert die Lage des Kreises dieses Netz. Von diesen selbst verfertigten Netzen sieht jedes anders aus. Man kann so das Gitter beim Trimmen mit dem Fotografierten identifizieren und etwaige Verwechselungen können dieses Stadium der Bearbeitung der Präparate nicht unbemerkt durchlaufen.

Die gute Definition des Platzes einer Zelle in den Planquadraten animiert dazu, eine Zelle während der Kultur ggf. über Tage hinaus mehreremals wieder aufzusuchen, so daß für das ultrastrukturelle Studium die Geschichte der Zelle in der Kultur bekannt ist. Auch Zellwanderungen können gemessen werden.

Die für diese Identifizierungsmethode erforderliche Sonderarbeit ist das Eingravieren des Netzes. Es gilt, mit Hilfe von Kreuztisch und Diamantschreiber (Zeiß oder Leitz) das Gitter in die genaue Mitte des Deckgläschens einzuritzen (Abb. 10). Das lernt aber jede Laborhilfe leicht. Unsere Spül-

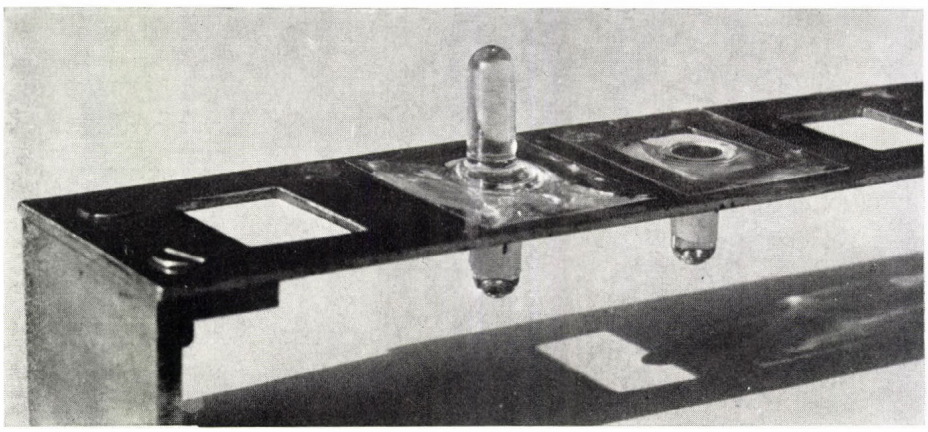


Abb. 12. 24 Stunden gehärtete Kapseln. Bei der einen ist der Kultur gegenüber auf die bloße Seite des Glases eine zweite Kapsel aufgesetzt

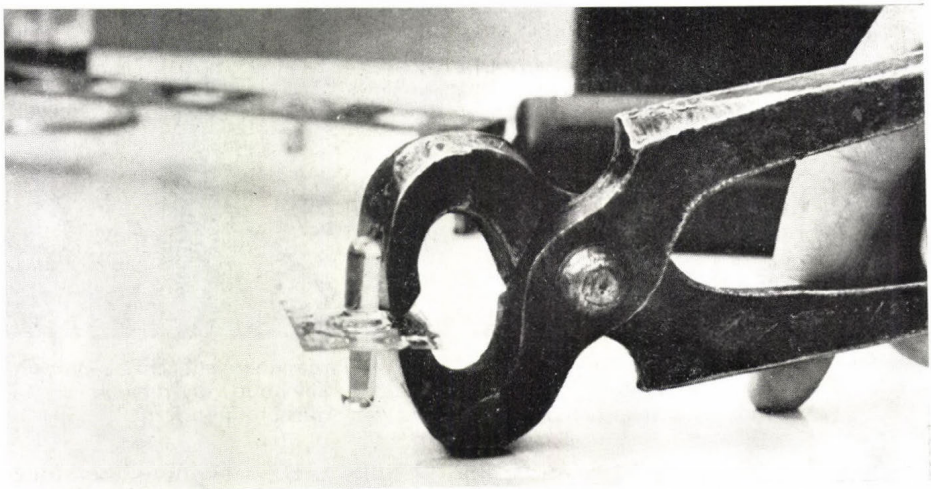


Abb. 13. Nach weiteren 48 Stunden Heißluftaufenthalt wird mit der Zange das die Kapsel überragende Glas mit Einbettungsmittel abgeknipst

frau verfertigt die 10–20 Stück für einen Kulturansatz in einer Stunde. Der Mikroskopiker selbst ist, wenn die Kultur schon mit einem Netz angelegt worden ist, von aller Markierungs- und Zentrierungsarbeit zwischen Beobachtung der Kultur bis zum Trimmen des Blockes befreit: das Deckgläs-

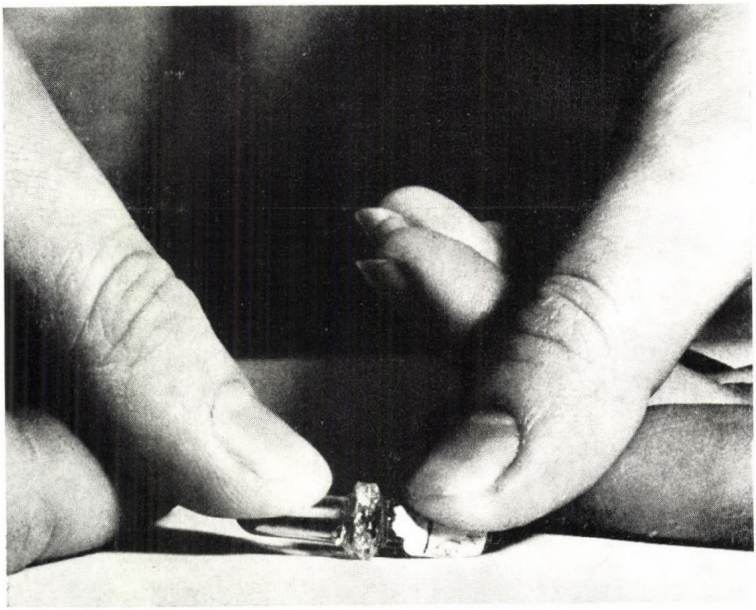


Abb. 14. Über dem etwas herausstehenden Deckglasrest als Drehpunkt lassen sich die Kapseln auseinanderbrechen. Es empfiehlt sich, zunächst unter Drehen mit geringem Druck zu beginnen

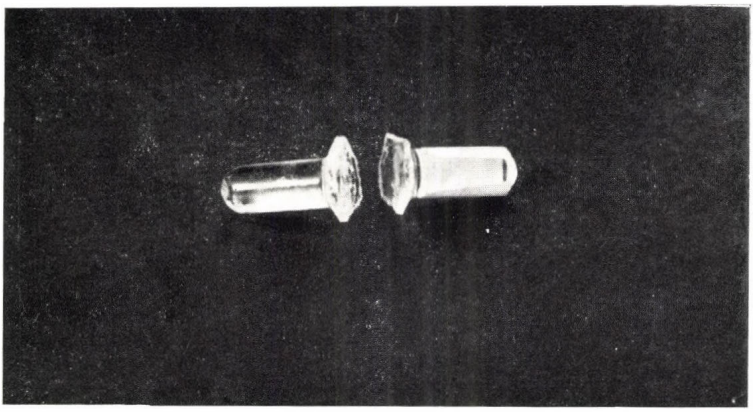


Abb. 15. Die etikettierte Kapsel enthält die Kultur. Das Glas geht beim Brechen mit auf die Hilfskapsel

chen wird, mit der für die Einbettung präparierten Kultur nach oben weisend, auf einen weißen Karton in eine Bleistiftskizze von Deckglasgröße gelegt. Die Diagonalen der Zeichnung treffen sich dort, wo das Netz liegt und leiten auf den richtigen Platz zum Aufsetzen der Kapsel (Abb. 11).

Auf die Geschichte der Kunstgriffe, wie man das Deckgläschen präparieren muß, um später das Einbettungsmittel wieder ablösen zu können, sei hier nicht näher eingegangen, da ein voll befriedigendes Verfahren noch allenthalben gesucht wird. Hat man wie Bloom (1960) nach Dr. H. Fernández-Moráns Vorschlag Kohle auf das Deckglas aufgedampft, so empfiehlt sich, besonders wenn die Kultur längere Zeit auf der Kohleschicht gewachsen war, 24 Std. nach der ersten Härtung bei 60 °C eine zweite Kapsel der ersten gegenüber auf die bloße Seite des Deckglases zu setzen (Abb. 12). Nach vollständigem Aushärten der beiden Kapseln, d. i. nach 72stündigem Heißluftschrankaufenthalt, zwickt man die überstehenden Deckglasteile mit einer Zange ab (Abb. 13) und bricht die beiden Kapseln über dem noch herausstehenden Rest auf einem Tisch auseinander (Abb. 14). Mit Regelmäßigkeit bleibt der Deckglasrest an der zweitaufgesetzten Kapsel haften (Abb. 15). Die Zellen liegen unmittelbar unter der jetzt freigewordenen Oberfläche des erhärteten Einbettungsmittels. Beim Trimmen sieht man von der Abbildung meist nur das Netz. Als Pyramidenspitze wählt man die Stelle des Netzes, an der im Übersichtsbild der Kultur die Zelle liegt.

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FINE STRUCTURE OF THE RETINAL PIGMENT EPITHELIUM IN ORGAN CULTURE

by

P. Röhlich and O. Török
1st Central Laboratory of Electron Microscopy and
Department of Histology and Embryology,
Semmelweis Medical University, Budapest, Hungary

For a long time it had been believed that the retinal pigment epithelium serves as a dark chamber for the visual process. However, as early as in 1879, Kühne pointed out that the pigment epithelium is necessary for the regeneration of the visual purple after bleaching. Further physiological and biochemical studies (Hubbard and Colman, 1959; Hubbard and Kropf, 1959; Dowling, 1960; Hubbard and Dowling, 1962) indicated that the pigment epithelium is involved in the metabolic cycle of rhodopsin being the site of reisomerization of the chromophore molecule. In addition, the specialized fine structure of the pigment epithelium (Porter and Yamada, 1960; Yamada, 1961, Bernstein, 1961; Dowling and Gibbons, 1962; Feeney et al., 1966; Matsusaka, 1967; Röhlich, 1967), especially the abundance of smooth endoplasmic reticulum could be interpreted satisfactorily only on the basis of pigment synthesis.

All these observations suggested that the retinal pigment epithelium must have an additional, perhaps more important, function than to shield photoreceptors from scattered light. A special nursing role of the retinal epithelium for the photoreceptor cells was the most probable hypothesis in this respect. We assumed that a close contact of the photoreceptors with the epithelium is a prerequisite for the normal development and maintenance of the specialized photoreceptor structure. In order to approach this problem, we have undertaken *in vitro* studies maintaining the neural retina and the pigment epithelium in organ cultures separately or in close contact with each other. The present work, which is the first part of this investigation, reports on the ultrastructural characteristics of the pigment epithelium maintained in organ culture.

Eyes of 11, 13, 15, and 18-day-old chicken embryos were dissected and the pigment epithelium was isolated either together with the scleral cartilage or separated from it. The epithelium was then placed on lens paper floating on fluid culture medium. Two media were used: one was a 1 : 1 mixture of bovine serum and chicken embryo extract, and the other one was Trowell's (1959) T8 medium supplemented with 15 per cent bovine serum. A total of 85 cultures were prepared, fixed and processed for electron microscopy on the 2nd, 3rd, 4th, 5th, 7th and 9th days of cultivation.

In healthy cultures the fine structure of the pigment epithelium was rather similar to that of chicken embryos of the same age. Apical cytoplasmic processes (Fig. 2) and a basal labyrinth (Fig. 3) were characteristic features of the epithelium in these cultures. The labyrinth began to appear focally at the cell contacts in the basal region of the epithelium and it was

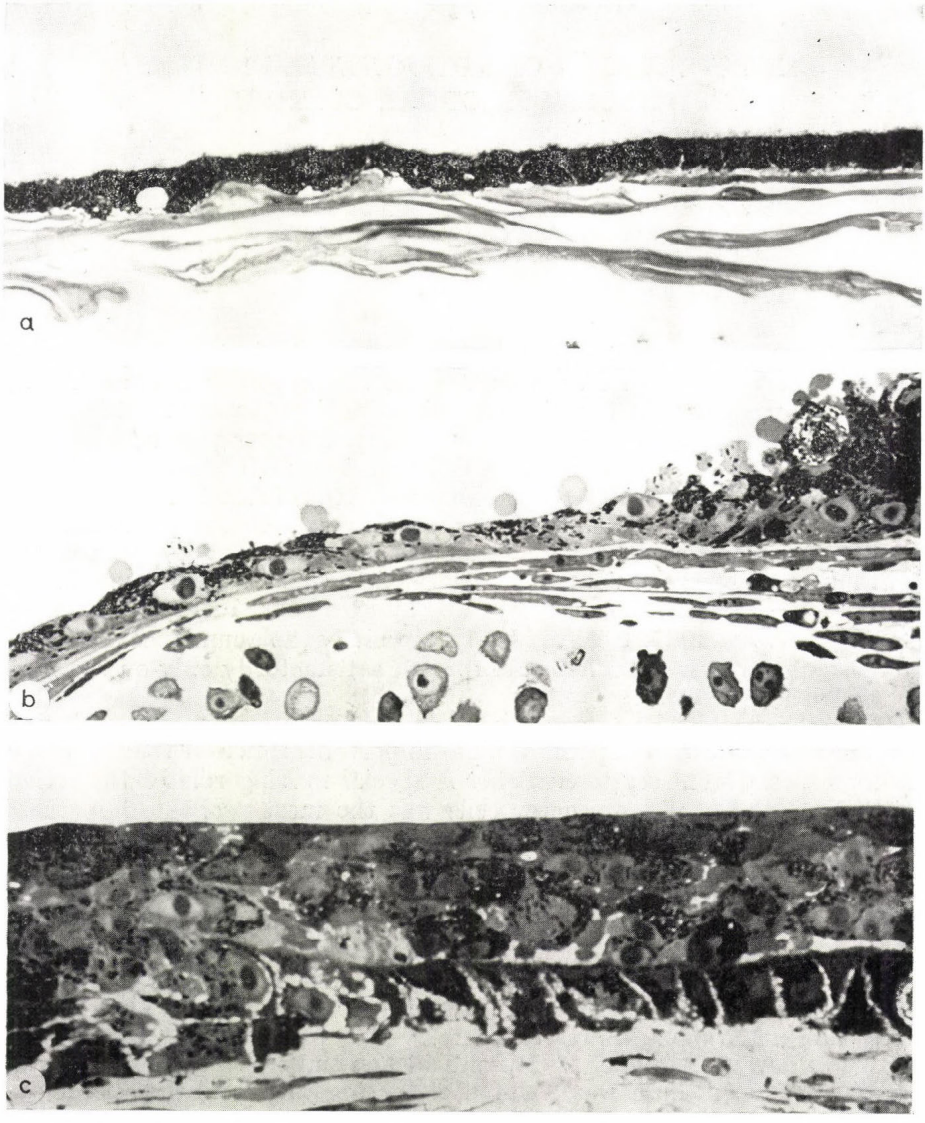
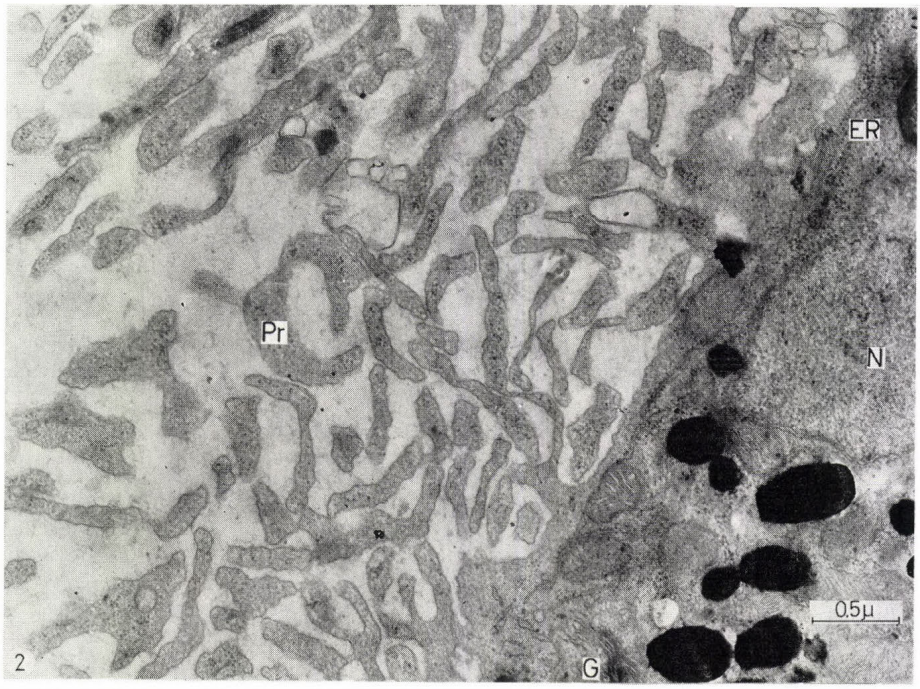


Fig. 1. (*a*) Light micrograph of the epithelial layer grown without cartilage on serum and chicken embryo extract. Semithin section. (*b*) Light micrograph of epithelial cultures grown on T8 medium. Note giant nucleoli, spherical cytoplasmic processes at the apical surface, and (*c*) multilayered appearance. Semithin sections

→

Fig. 2. Apical surface of the epithelium. Pr, long cytoplasmic processes; ER, ergastoplasmic cisterna; G, Golgi apparatus; N, nucleus

Fig. 3. Basal region of the epithelial cells. N, nucleus; J, junctional structure; M, mitochondria; BL, basal labyrinth; BM, basement lamella; C, collagen fibrils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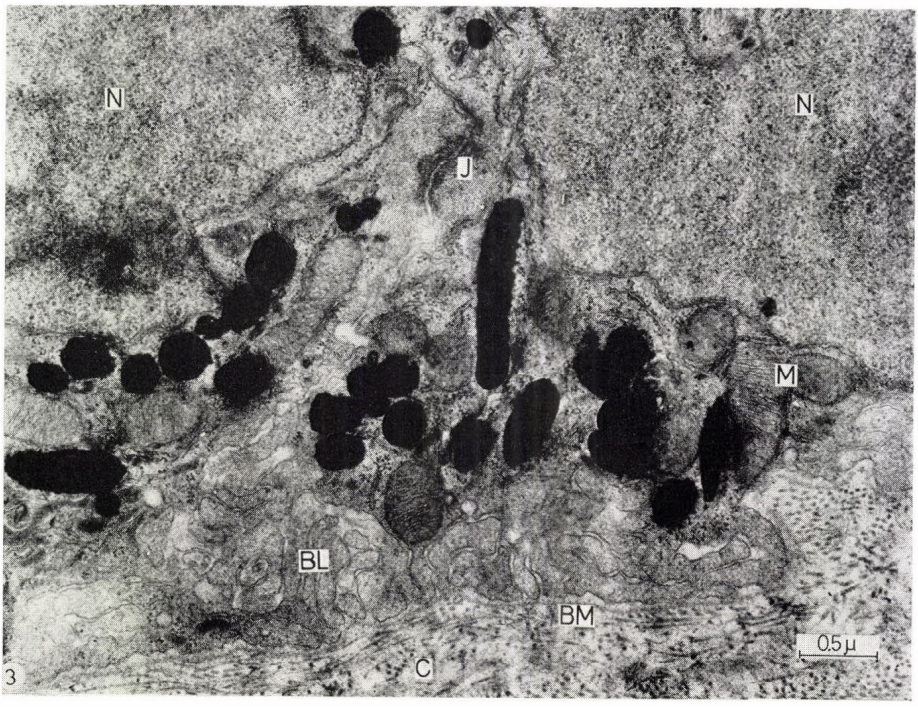
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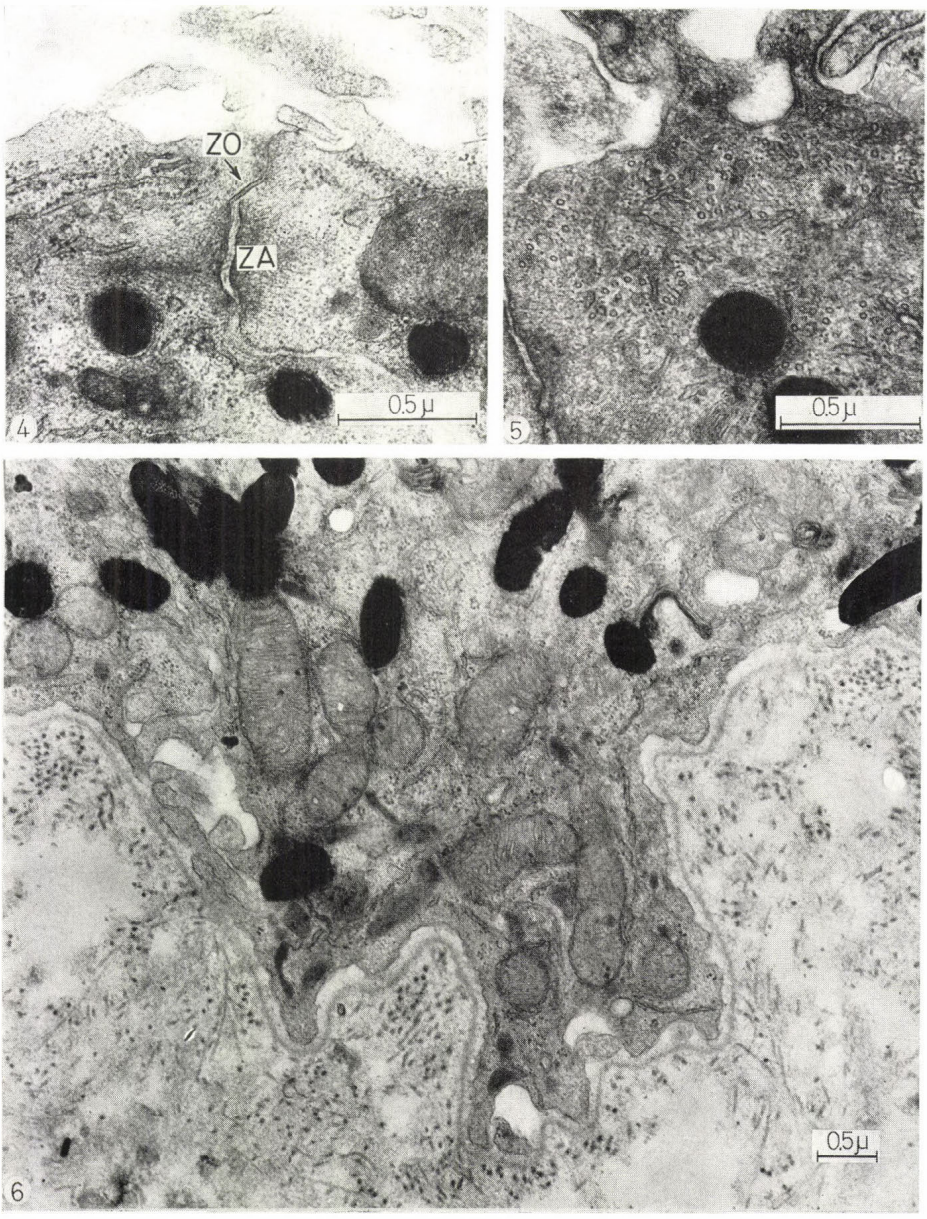
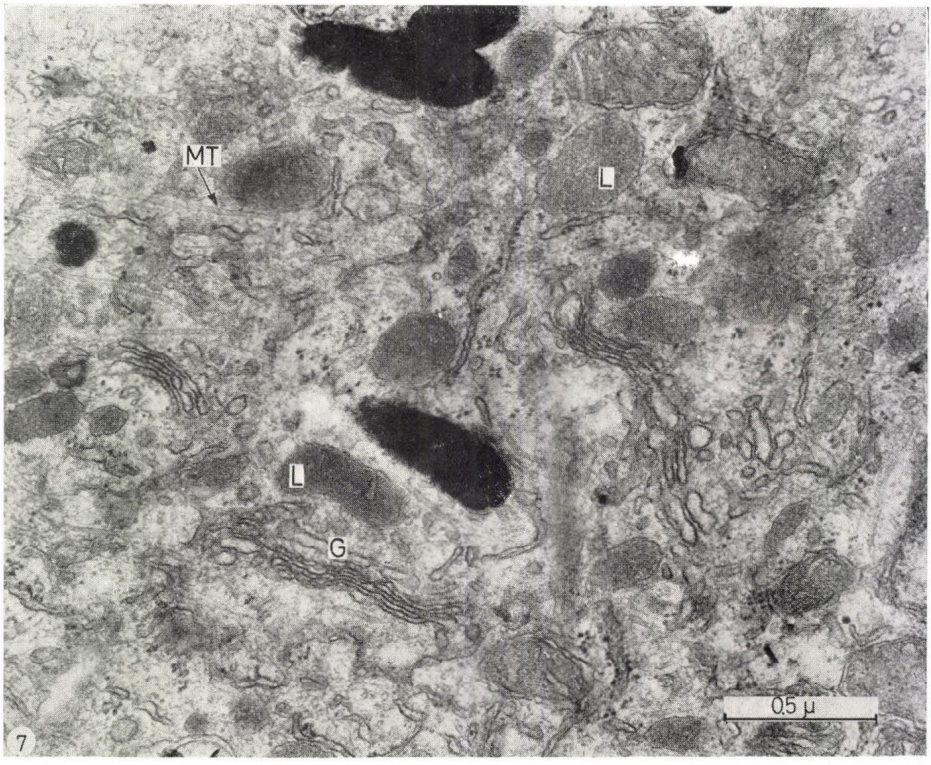


Fig. 4. Cell junction between epithelial cells. ZO, tight junction; ZA, zonula adhaerens

Fig. 5. Cytoplasmic microtubules and filaments in cross section

Fig. 6. Basal process of the epithelial cell. The process contains mitochondria and forming progranules



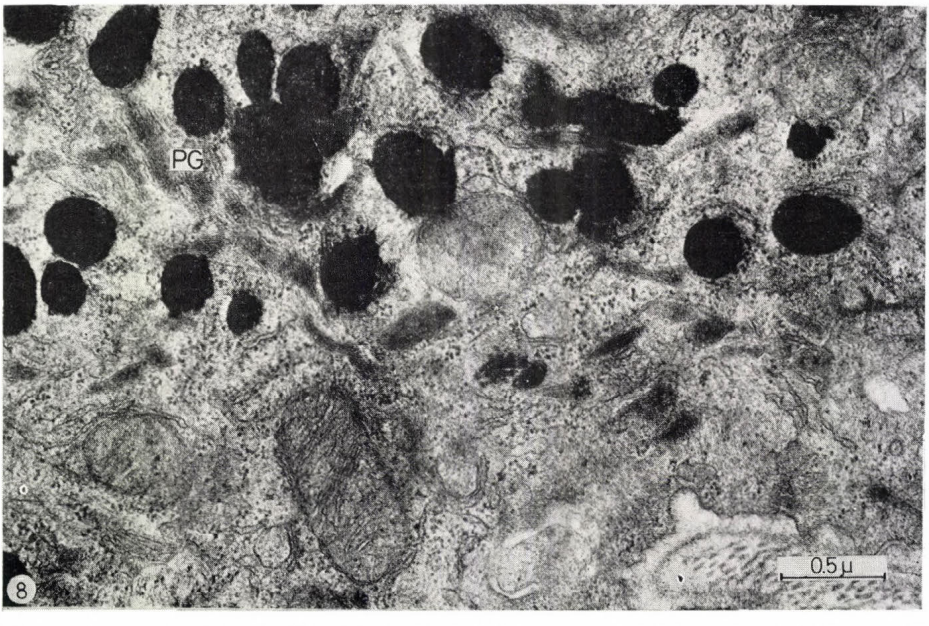


Fig. 7. Lysosome-like bodies (L) in the vicinity of the Golgi apparatus (G); MT, microtubule

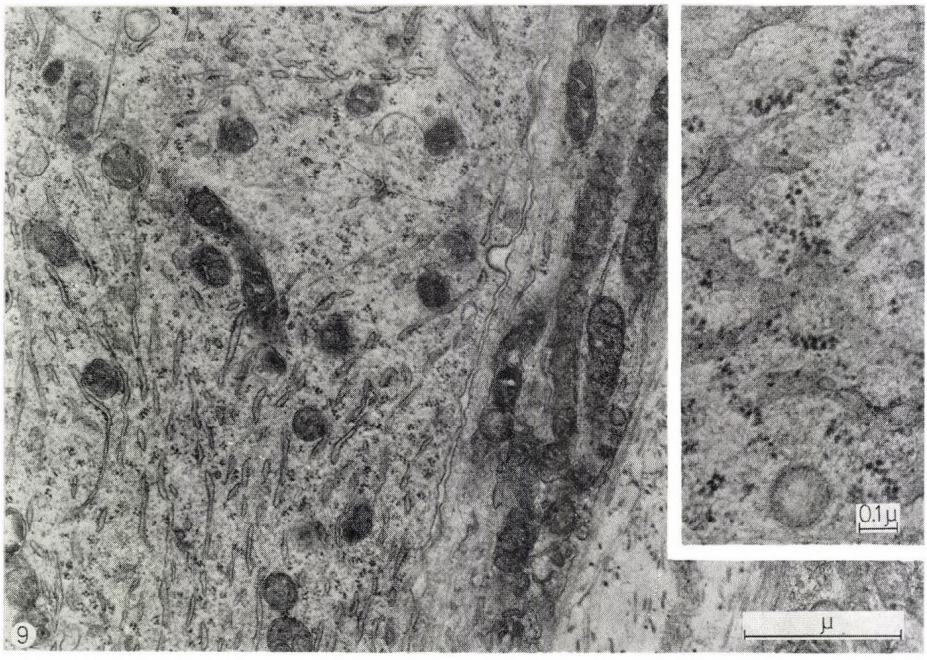
Fig. 8. Progranules (PG) in the basal region of the pigment cell

well developed in cultures taken from older embryos. The epithelium was separated from the underlying connective tissue by a basement lamella (Figs 3 and 6). Junctional structures (Fig. 4) were observed in the apical region and consisted of a tight junction and a zonula adhaerens. Typical desmosomes were not present. The epithelial cells showed a certain polarity. The basal region of the cell (Fig. 3) was occupied by the basal labyrinth and contained most of the mitochondria. The nucleus was found in the central or somewhat apical region and the few ergastoplasmic cisternae were generally located above the nucleus. The Golgi apparatus could be found in the supra- or paranuclear area. The smooth endoplasmic reticulum was not yet developed in these cultures, similarly to the pigment epithelium of the *in vivo* material. Elongated pigment granules were scattered in the cytoplasm but they were fewer in number than in the original material.

About one half of the epithelial cultures showed some deviations from the general pattern described above. The basal labyrinth was sometimes underdeveloped and the cells sent long basal processes into the connective tissue (Fig. 6). The processes were usually devoid of pigment granules and contained mitochondria and a few ER cisternae. Disintegrating pigment granules and newly formed proganules (Figs 6 and 8) were occasionally observed. In about one third of all pigment cultures, the epithelial cells contained a varying number of lysosome-like dense bodies which were often found in the vicinity of the Golgi apparatus (Fig. 7) or filled all free spaces in the cytoplasm. It must be mentioned that lysosomes are steady constituents of the differentiated pigment epithelium and participate in the digestion of phagocyted outer segment material of the photoreceptors.

The above findings indicate that the pigment epithelium can be maintained successfully in organ culture and about half of the cultures shows a well-preserved normal appearance of the epithelium. This was a good starting point for the second part of our investigations in which neural retina and pigment epithelium are maintained in organ culture in close contact with each other. Preliminary results indicate that outer segments will successfully develop when the retina is cultured in contact with the epithelium. In contrast, retinae from which the pigment epithelium had been previously separated are practically void of outer segments.

Cultures grown on the T8 medium showed an interesting transformation of the pigment epithelial cells. The cells became more voluminous and contained giant nucleoli when seen in the light microscope (Fig. 1*b*). The cells frequently proliferated and formed a multilayered epithelium (Fig. 1*c*) resembling the stratified squamous epithelium. Basal labyrinth and apical processes were absent. (Fig. 9); the smooth apical surface bulged out occasionally to form cytoplasmic blebs apparently separating itself from the epithelial surface (Fig. 13). Giant nucleoli (Figs 11 and 12) often occupied one third or one half of the nuclear area, the rough ER cisternae were generally degranulated (Figs 9 and 10) and many free ribosomes and polysomes were found in the cytoplasm. The latter frequently showed a helical configuration (Fig. 9, inlet). The number of cytoplasmic filaments and microtubules greatly increased and large areas of parallel filaments were frequently observed. (Fig. 15). Pigment granules gradually disappeared from the cells (Fig. 9), mitochondria became smaller and more numerous.



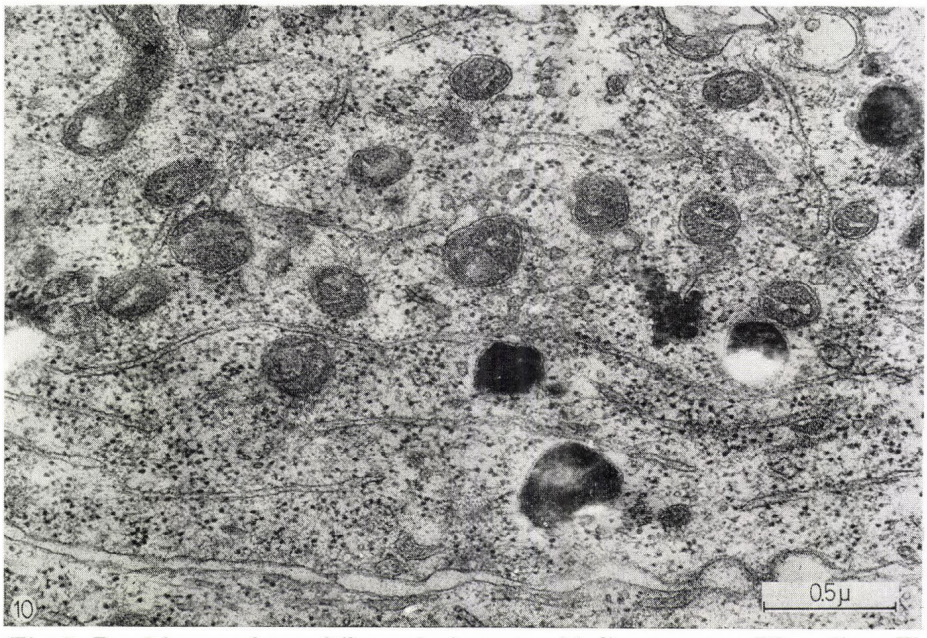


Fig. 9. Basal layers of a multilayered pigment epithelium grow on T8 medium. The cells are devoid of pigment granules. Mitochondria are relatively small, cisternae of the endoplasmic reticulum are degranulated and many helical polysomes are seen in the cytoplasm. Basal labyrinth has not developed. Inlet: helical polysomes in cells grown on T8 medium

Fig. 10. Degranulated ER cisternae and free ribosomes. (T8 medium)

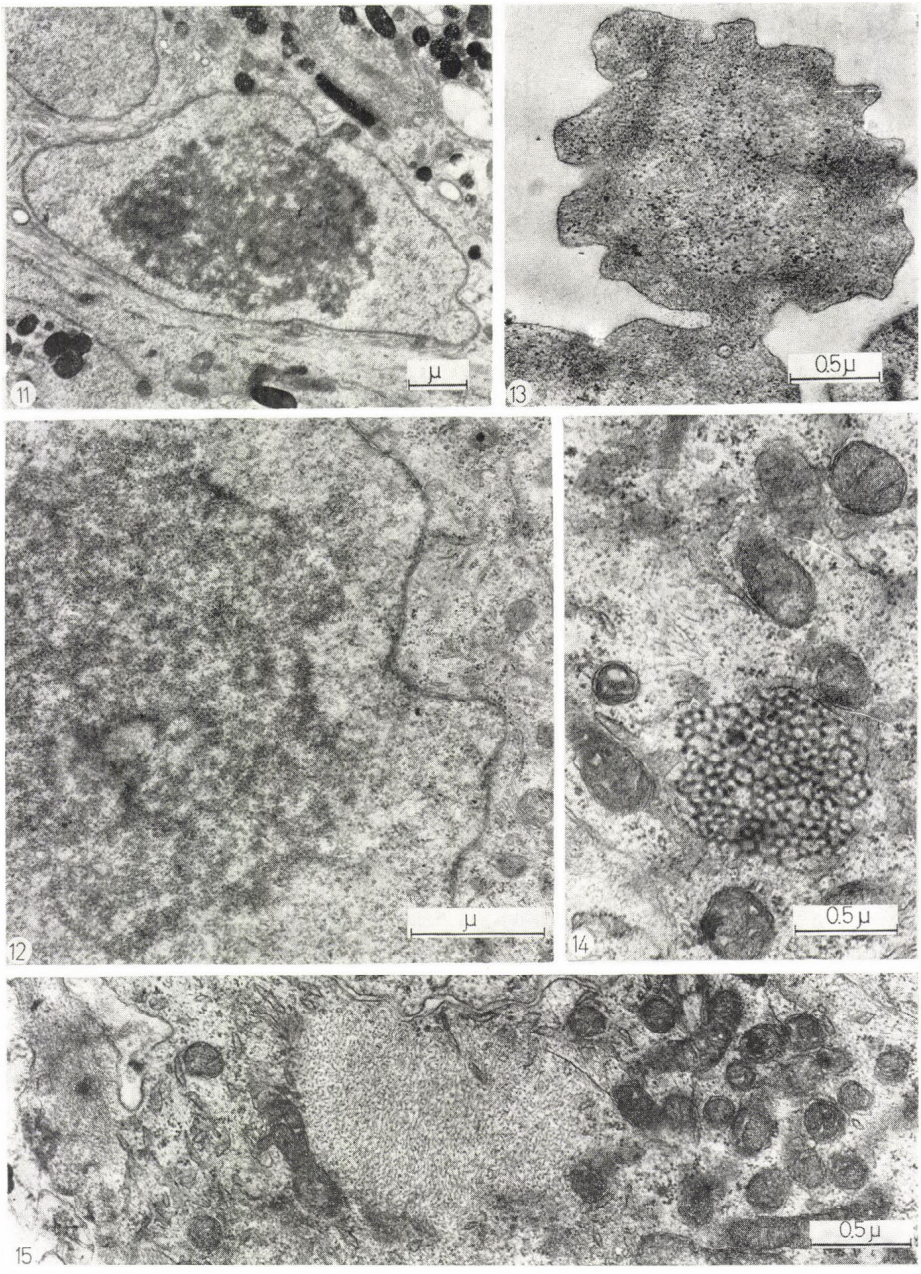


Fig. 11. Giant nucleolus (T8 medium)
Fig. 12. Giant nucleolus with higher magnification (T8 medium)
Fig. 13. Spherical cytoplasmic process at the apical surface of the epithelium (T8 medium)
Fig. 14. Reticular body in the cytoplasm. (T8 medium)
Fig. 15. Accumulation of cytoplasmic filaments (T8 medium)

Sometimes bodies of a reticular structure appeared free in the cytoplasm (Fig. 14), their nature and origin is unknown.

It is surprising to find such a profound alteration of cell fine structure in cultures grown on the T8 medium, since Trowell (1959) recommended T8 as a simple, suitable medium for organ cultures. Franks (1961) observed that the prostate epithelium grown on the T8 medium showed a striking hyperplasia which was due to insulin present in the medium. Insulin has been frequently used in tissue cultures to promote cellular proliferation. The present fine structural findings indicate that insulin profoundly affects regulation of cell metabolism, possibly in the DNA–RNA-protein system. Further studies in this laboratory have been initiated to analyse the cellular effect of insulin on different cell types and with different methods.

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Symp. Biol. Hung. 11, pp. 137–143 (1972)

THE ULTRASTRUCTURE OF DNP SYSTEMS IN TISSUE CULTURES AS REVEALED BY THE POLARIZATION MICROSCOPE

by

M. Kellermayer and K. Jobst
CENTRAL CLINICAL LABORATORY OF THE MEDICAL UNIVERSITY OF PÉCS, HUNGARY

Optical anisotropy, i.e. birefringence, is the sign of orientated structural organization in macromolecular systems. This statement is, however, not quite true for all biological structures, e.g. for the nucleus. On the other hand, it would not be right if we assumed that there was an unorientated structure or substrate in the background of the elective function of the nucleus. This is made improbable by the weak birefringence of giant chromosomes, sperms and some unicellular organisms (Schmidt, 1938; 1941; Frey-Wyssling, 1943) and also by the intensive negative birefringence of DNA, the chief structural component of isolated nuclear substance (Schmidt, 1938; Jobst, 1962).

We approached the problem of the optical isotropy of nuclei from the aspect of a special, intermolecular relationship between intranuclear DNA and protein.

In contrast to earlier investigations of this kind, our present experiments were performed on tissue cultures. We used Romhányi's 'TPS' method, i.e. toluidine blue staining followed by precipitation (Romhányi, 1963). The preparations were stained with an 0·1 per cent solution of toluidine blue in pH 3·2 veronal buffer for 10 min, then the dye was precipitated with 2 per cent potassium ferricyanide, and the preparations were mounted in gum arabic. Essentially, we were led by the observation that the anisotropy of structures showing very mild birefringence in the unstained state can be greatly enhanced by means of the orientated association of certain dye molecules (White and Elmes, 1952). Thus after rivanol or toluidine blue staining followed by precipitation with potassium ferricyanide, DNA fibres showed a considerably increased birefringence (Romhányi and Jobst, 1957; Jobst, 1962). Therefore, we assumed that the dye molecules were bound to DNA thus producing intranuclear DNA anisotropy, which, up to now, had been hardly measurable. This assumption has now been confirmed by our experiments.

The present paper is a report on the polarization optical (PO) observations made on the submicroscopic structure of intact cells and on that of isolated nuclei.

The PO method allows the direct study of the optical properties of cells and nuclei in intact cells of monolayer cultures, without the artificial effects leading to anisotropy, and without previous preparation or chemical fixation. This is an essential requirement because, according to our observations, nuclear birefringence is changed fundamentally, though not uniformly, by the different fixatives (Kellermayer and Jobst, in press). Thus,

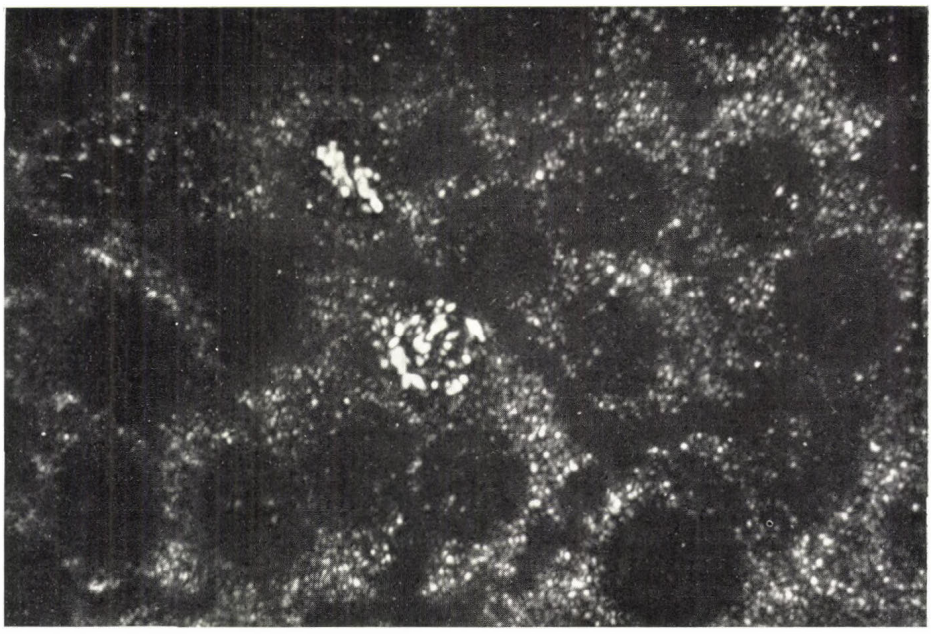

Fig. 1

in the 48 to 72-hour cover glass cultures of HeLa cells interphase nuclei as well as mitotic chromosomes become isotropic after fixation with 4 or 10 per cent neutral formalin. After fixation with 1 per cent osmium on the other hand, mitotic figures, and also resting nuclei, become birefringent. Thus, the two different fixatives produce basically differing PO pictures. The PO pictures were more differentiated in the preparations fixed in a 3 : 1 mixture of alcohol and glacial acetic acid, and in freeze-dried or only air-dried ones. Here, in agreement with the observations made on unstained preparations, only the mitotic figures were birefringent, while the interphase nuclei remained isotropic (Fig. 1). This birefringence could be followed from the prophase to the late telophase, however, it disappeared after separation of the daughter cells (Kellermayer et al., 1970).

In preparations of tissue cultures isolated by treatment with hypotonic saline and stained with the TPS method, the orientation and structure of the DNA of chromosomes can also be easily studied with the present method (Jobst and Kellermayer, in press).

In addition to the PO pictures of the preparations fixed by drying, cytophotometric measurements also indicated that the association of the dye molecules in the DNA of resting nuclei differed from their association in the DNA of mitotic chromosomes. The absorption peak of the resting nuclei stained with the TPS method was at 590 mμ, while that of the metachromatic dividing forms at 550 mμ (Kellermayer et al., 1970).

Summing up: (1) When using chemical fixatives one must reckon with submicroscopic changes in the structure of nuclear DNP–DNA systems, which can be easily followed by PO methods. The freeze-dried or air-dried

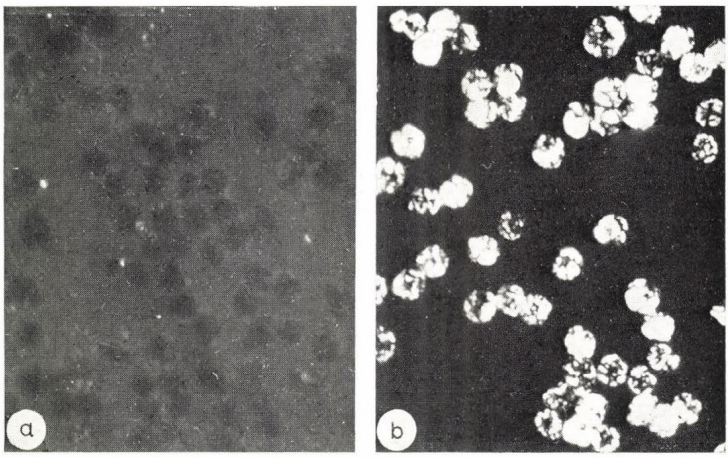

Fig. 2

preparations stained with the TPS method show the same PO picture as the nuclei of unstained preparations. Thereby the difference in structure between resting and mitotic nuclei is reflected also in the PO picture. Therefore, in our further experiments we only used fixation by drying.

(2) In addition to the condensation and heterochromatinization of the chromatin substance the change in structure of the DNP–DNA system, i.e. the birefrigence of dividing nuclei, could be demonstrated and measured polarization optically in the mitotic phase.

Although in this case the PO method has undoubtedly demonstrated a basic difference in structure between resting and dividing cells, the method failed to detect a difference in chromatin structure among the cells in the different phases of the mitotic cycle on the one hand and the chromatins of the resting nucleus on the other. On the basis of the functional differences between condensed heterochromatin and diffuse euchromatin (Frenster, 1965), a difference in the structural organization of DNA inside the single parts of the resting nucleus was to be assumed. Therefore, we examined this question more thoroughly on isolated nuclei.

We isolated thymus lymphocyte nuclei with Allfrey's method (Allfrey et al., 1964). In the isotonic 0·25 M sucrose used for isolation the lymphocytes were isotropic after staining with the TPS method (Fig. 2*a*). If, however, the nuclei were transferred into a medium of 120 mEq per 1 or higher sodium or potassium ion concentration, e.g. into Hanks' solution, they would become intensively birefringent (Fig. 2*b*). This phenomenon was reversible. When placed back in sucrose, the birefringent nuclei again became isotropic, and vice versa (Jobst and Kellermayer, 1967). By way of explanation we assumed that, simultaneously with the cation concentration of the surrounding medium, intranuclear ion concentration also varies. In response to this, ion dissociation of DNP takes place, and the DNA molecules are liberated from their protein binding, which produces an orientated association of the dye molecules with DNA. Therefore, such

nuclei are birefringent. In sucrose, intranuclear electrolyte concentration decreases, and DNP is reconstructed; the nuclei are isotropic.

In order to confirm our assumption we determined the potassium and sodium ion concentration of the nuclei isolated in sucrose and in Hanks' solution. The potassium and sodium ion concentrations of the nuclei isolated in Hanks' solution were both higher than those in sucrose; however, in contrast to the nearly twice as high potassium ion concentration we measured an ion concentration 20 times as high in the case of sodium (Table 1). These data supported our assumption that the reversible anisotropy of isolated lymphocytes can be explained on the basis of the ion concentration dependent dissociation of DNP.

TABLE 1

	n	μEq/g dry weight		μEq/ml H_2O		Dry weight %
		Na	K	Na	K	
Thymus nuclei in sucrose solution	26	16·3 ±5·4	186·3 ±50·4	5·0 ±1·4	59·2 ±12·3	24·2 ±2·5
Thymus nuclei in Hanks' solution	31	463·6 ±150·0	321·3 ±57·8	109·9 ±43·2	77·5 ±8·5	19·2 ±1·7

	Na	K, μEq/ml
Sucrose solution	—	—
Hanks' solution	142–146	5–5·4

We did, however, not succeed in distinguishing a discrete chromatin structure in the inactive nuclei of lymphocytes. We, therefore, extended the examinations to larger HeLa cells, which were more suitable in many respects, and in which the isolation of nuclei was performed with the 0·1

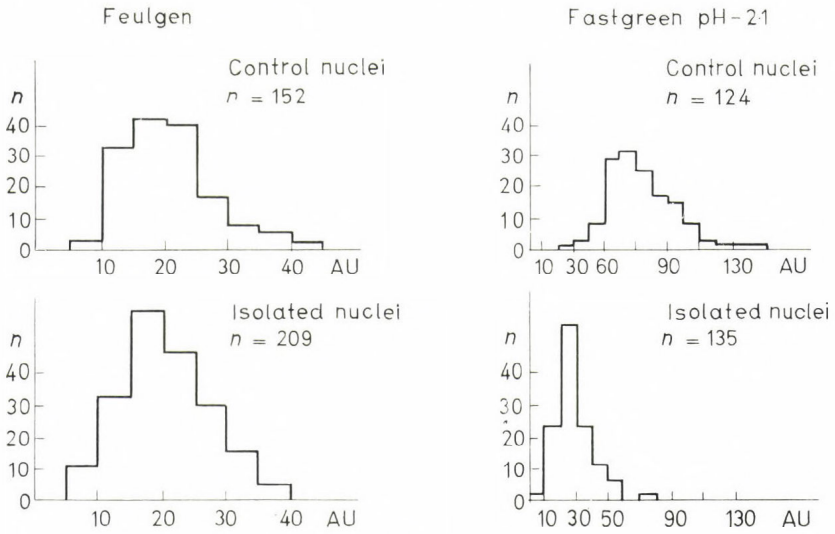

Fig. 3

per cent non-ionic detergents NP40 or Triton X100 (Kellermayer and Jobst, 1971). In order to prepare a solution of the detergent, we mixed 0·145 M NaCl and 0·145 M KCl in different proportions with 0·25 M sucrose in such a manner that the concentration of sodium and potassium varied between 0 and 145 mEq per litre by a factor of 5 mEq. After discarding the nutritive fluid, the above-described isolating fluid was poured on the cover glass culture. Occasionally, the detergent was dissolved in Hanks' solution. Cytolysis was continued at room temperature for 20 to 35 min, then the preparations were rinsed with a detergent-free solution of the same ion concentration, which was followed by drying, and staining with the TPS method.

In order to detect a possible interaction between the isolating medium and nucleolar DNP, and also the dissolving effect of the former, we determined the DNA and total protein content of the nuclei isolated with a detergent. Cytophotometry, according to Feulgen, showed that the DNA content of the nuclei isolated in Hanks' solution did not change, but their total protein content (FG pH 2·1) decreased by nearly 60 per cent (Fig. 3).

Polarization microscopy showed the nuclei isolated in sucrose to be isotropic (Fig. 4*a*). After isolation with Hanks' solution the whole nucleus showed roughly granular, intensive birefringence (Fig. 4*c*). The PO picture of the HeLa nuclei observed in the course of isolation with the two different detergents was, in every respect, the same as that described in the lymphocytes: only the nuclei isolated in electrolytes, e.g. in Hanks' solution, were birefringent. We believe that this optical effect is related to the heterochromatic structure of the nucleus. When the ion concentration of the solution was decreased from 144 m Eq per litre to 120 mqE per litre, the PO picture did not change. On the other hand, between 80 and 100 mEq per litre only perinucleolar heterochromatin remained birefringent (Fig. 4*b*).

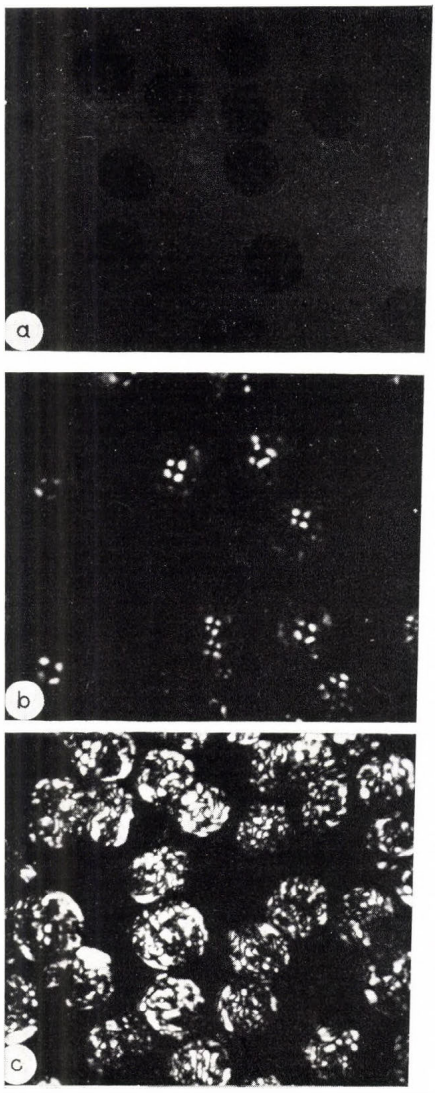

Fig. 4

The PO picture showed no alteration after RNase digestion. After DNase digestion, on the other hand, the birefringence of this special structure disappeared, which also proves the presence of DNP micelles arranged circularly round the nucleoi (Caspersson, 1950; Caspersson et al., 1963; McConkey and Hopkins, 1964; Busch and Smetana, 1970). At cation concentrations of 70 and 80 mEq per litre, perinucleolar heterochromatin also becomes isotropic. However, inside the nucleus the fine, dust-like birefringence, which we identified as that of the euchromatin structures of the nuclei, continues to remain recognizable.

The described PO effect can be equally elicited with a pure NaCl or KCl solution, or a mixture of the two. Thus, it is not the quality but the concentration of the cation that is responsible for the PO change. Below a cation concentration of 70 mEq per litre we saw no birefringence in the isolated nuclei: the whole nucleus was isotropic, like a nucleus isolated in sucrose.

To sum up our PO observations made on isolated nuclei: in the nuclei anisotropy, in other words the structural organization of chromatin, i.e. of DNP–DNA, is a function of the cation concentration of the medium surrounding the nuclei. It has been proved that the genetic activity of these chromatin structures is also a function of the cation concentration of the milieu (Kroeger, 1963; Lezzi, 1969). As yet we cannot tell whether or not there is a relationship between the PO picture observed by us and the functional data, and if there is one, what it is like. At any rate, it is remarkable that the cation sensitivity of euchromatin, which is genetically the most active of chromatins, and that of perinucleolar heterochromatin are greater than that of heterochromatin. The former two become birefringent at a lower ion concentration (at 70–110 mEq per litre) than do the inactive heterochromatic structures of the nucleus. These findings also seem to show that there must be a difference in the dissociation constants of the DNP of these nuclear chromatin structures.

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Symp. Biol. Hung. 14, pp. 145–159 (1972)

ULTRASTRUCTURE OF PLANT CELLS GROWING IN SUSPENSION CULTURE

by

H. E. Street, M. R. Davey* and B. Sutton-Jones**
BOTANICAL LABORATORIES, UNIVERSITY OF LEICESTER, UNITED KINGDOM

The study of ultrastructure has its greatest interest when it represents a component of a more comprehensive approach to cellular function. A number of studies have now been made on the changing patterns of growth and of metabolism of batch-propagated plant cell suspension cultures and hence we can fairly closely correlate features of their fine structure with their known physiology. This particularly applies to cultures of cells of the English sycamore (*Acer pseudoplatanus* L) which form the theme of the present paper.

TECHNIQUES OF CULTURE AND ELECTRON MICROSCOPY

Culture techniques, growth patterns and changes in metabolism. Excised young shoots of sycamore transferred to the surface of a suitable solidified medium generate a wound callus by proliferation from the existing cambium (Lamport, 1964). This callus can be subcultured and maintained indefinitely on a synthetic culture medium (Stuart and Street, 1969). When transferred to a shaken liquid medium this callus readily breaks up to give a growing suspension culture composed of free cells and small cell aggregates (Henshaw et al., 1966) and this suspension can be grown indefinitely in batch culture by regular subculture. The standard batch culture procedure involves 250 ml wide-mouthed Erlenmeyer flasks containing 70 ml culture medium incubated at 25 °C on a horizontal platform shaker (130 r.p.m. 5 cm throw) and initiated by transfer of an aliquot (1–10 ml) of a 28-day-old culture.

The growth of such cultures in terms of increase in cell number per unit volume of culture conforms closely to the generalized curve shown in Fig. 1. When sycamore cultures are initiated at an initial cell density of ca. 10^5 cells per ml the lag phase is of about 3 days duration, the exponential phase is transient usually involving not more than a $\times 4$ increase in cell number and stationary phase is reached after 15–20 days of incubation. By stationary phase the cultures contain $3–4 \times 10^6$ cells per ml. The average volume per cell is at a minimum at the end of the short exponential growth phase and reaches its maximum value during the phase of progressive deceleration of growth rate. While cell division is rapid the proportion of

Present addresses:
* Department of Botany, University of Nottingham, United Kingdom.
** School of Biological Sciences, University of East Anglia, Norwich, United Kingdom.

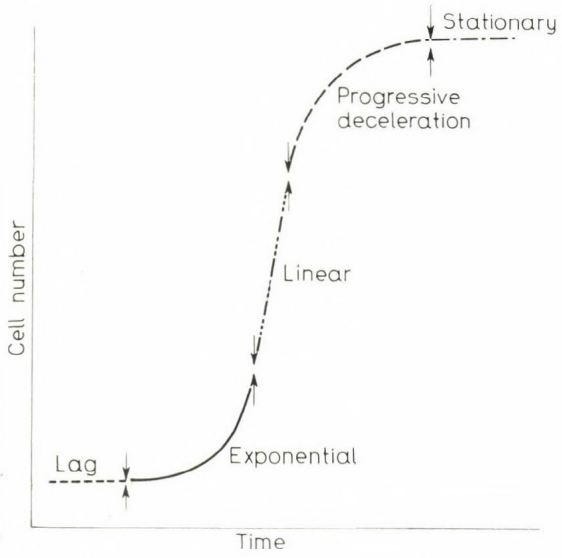


Fig. 1. Model curve relating cell number per unit volume of culture to time in a batch grown plant cell suspension culture. Growth phases labelled

the cells in aggregates and the average number of cells per aggregate both rise; the aggregates break up during the phase when cell expansion is dominant and the stationary phase culture has a high content of free cells. Dry weight per cell also declines during the phase of rapid cell division, reaches a maximum in early stationary phase and declines if stationary phase is prolonged due to carbohydrate starvation. Associated with this growth pattern there are changes, in the physiological activity of the cells.

During the lag phase and overlapping into the phase of exponential growth there is progressive rise in the protein content per cell (up to $\times$ 4 the initial level of the stationary phase cells) (Givan and Collin, 1967) and in RNA per cell (up to 3·5 the initial level) (Short et al., 1969). Unpublished data show that peaks of protein and RNA synthesis occur at this time. At this stage the cells are active in amino acid synthesis (Givan and Collin, 1967; Simpkins and Street, 1970) and in the synthesis and interconversion of nucleotides (Brown and Short, 1969). These changes are accompanied by a corresponding rise in O_2 uptake per cell ($Qo_{2(N)}$ remains about constant). Measurements of the C6/C1 ratio (Bloom and Stetton, 1953) indicate that during this initial phase of culture the pentose phosphate pathway makes a significant contribution to the total O_2 uptake (C6/C1 ratios close to 0·4) whereas in the later phases of the growth cycle of the culture the O_2 uptake is almost entirely by the E–M–P pathway (C6/C1 ratios close to unity) (Folwer, 1971). These inferred changes in the activity of these two respiratory pathways correlate well with the levels of the key regulatory enzymes involved (phosphofructokinase, glucose-6-phosphate dehydrogenase and transketolase). The significance of the pentose pathway may be the production of the NADPH required for biosynthetic reactions, (AP Rees and Beevers, 1960; Ragland and Hackett,

1961). Work with mitochondria isolated from these cultured cells (Wilson, 1971) indicates that they change in their sensitivity to cyanide inhibition as culture growth proceeds, the cyanide-insensitive component reaching a transient peak early in the growth cycle when the cells are presumably calling for short carbon skeletons for biosynthesis. The synthesis and release into the culture medium of hemicellulose material proceeds most actively once cell division is initiated and declines sharply as the phase of progressive deceleration of cell division is approached (Street et al., 1968).

All these observations indicate an initial period of intense biosynthesis, leading to a phase of macromolecular synthesis and this in turn to a decline in metabolic activity per cell associated with the cessation of cell division in the cultures. At this time cell expansion becomes dominant and associated with this there occurs a rapid increase in cell volume and an increase, while carbohydrate remains externally available, of dry weight per cell (of which starch becomes an important component). This definable pattern of growth and metabolic activity receives pictorial confirmation by the fine structural data now to be presented.

Electron microscopy. The cells were collected as an 8 mm layer on a single layer of nylon sifting cloth (nylon shirt material which had been laundered several times was very satisfactory!) used to close one end of a glass 'transfer tube' 16 mm in diameter and 50 mm long (Sutton-Jones and Street, 1968). The drained cells were submitted to further treatments by standing the transfer tube in flat-bottomed specimen tubes (80×22 mm) containing fixative, dehydrating and impregnating solution. Cells were pre-fixed in 0·6 per cent glutaraldehyde in 0·1 M sodium cacodylate (pH 7·2) for 16 h washed with cacodylate buffer and post-fixed in 1 per cent osmium tetroxide for 1·5 h at pH 7·2. After a second 30-min wash dehydration was effected using graded ethanol/water mixture, followed by embedding in methacrylatestyrene (Mohr and Cocking, 1968). During the final dehydration stage, the material was pre-stained in a millipore filtered solution of 1 per cent uranyl acetate in absolute ethanol for 1 h. Fixation and dehydration was carried out at 4 °C. Following infiltration, cells were transferred to No. 2 size gelatin capsules (Parke-Davis Ltd.), polymerized at 55 °C and sectioned in a Tesla Ultramicrotome BS 490 with glass knives. Sections were collected on celloidin-coated Polaron C2000 copper grids, stained with Reynolds' lead citrate for 5 min and examined in a Siemens Elmiskop 1A electron microscope, using an accelerating voltage of 80 k, a 200 μm condenser aperture and a 50 μm objective aperture (Davey and Street, 1971). Prior to the development of this technique araldite was used as the embedding medium (Sutton-Jones and Street, 1968); the use of methacrylate-styrene considerably shortened the impregnation phase and the sections so prepared gave better contrast on staining.

THE FINE STRUCTURE OF THE CELLS OF SYCAMORE SUSPENSION CULTURES

The stationary phase cells used to initiate the cultures are highly vacuolated; they have a large central vacuole and often smaller vacuoles within the cytoplasm. The cytoplasm forms a thin peripheral lining within the cellulose

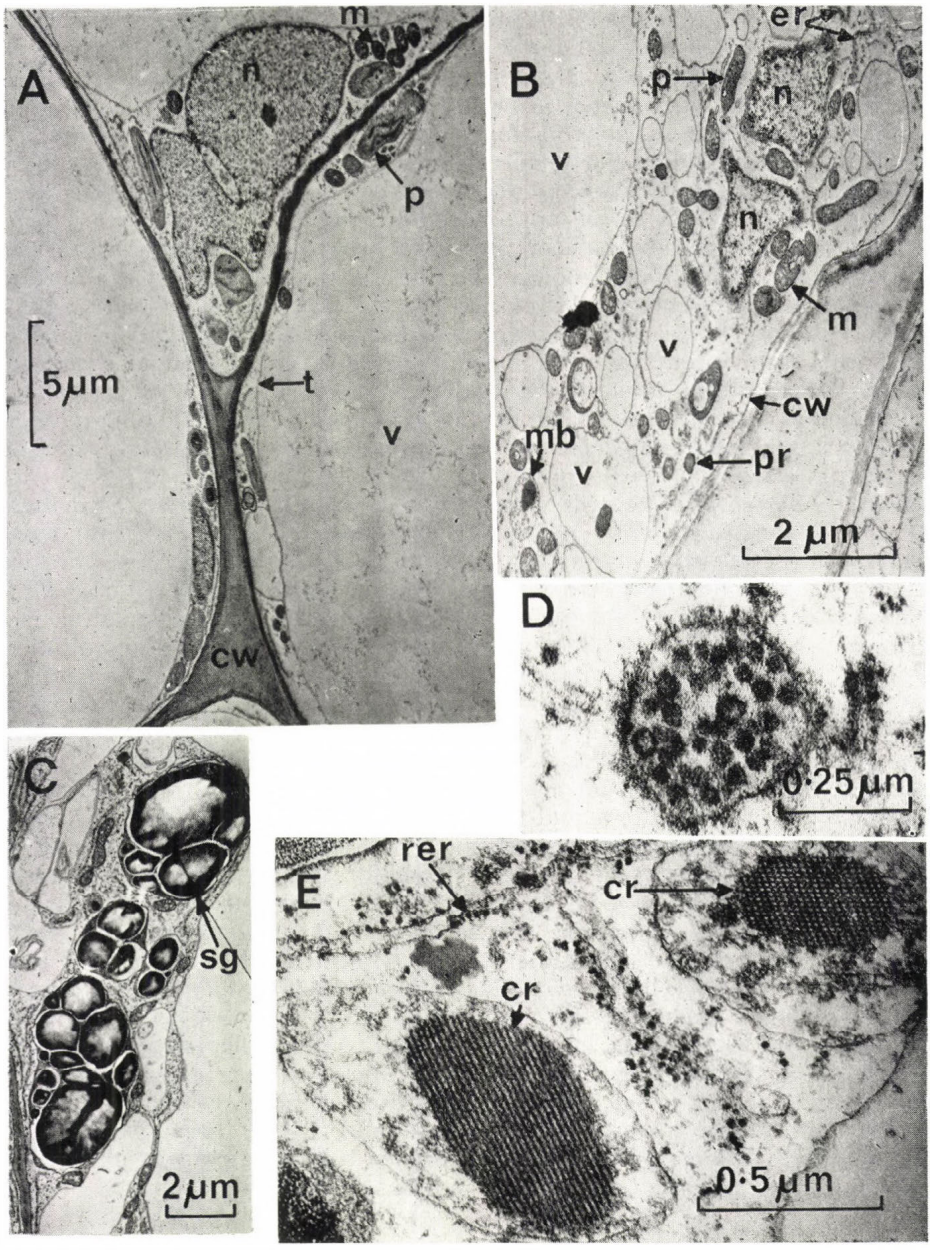


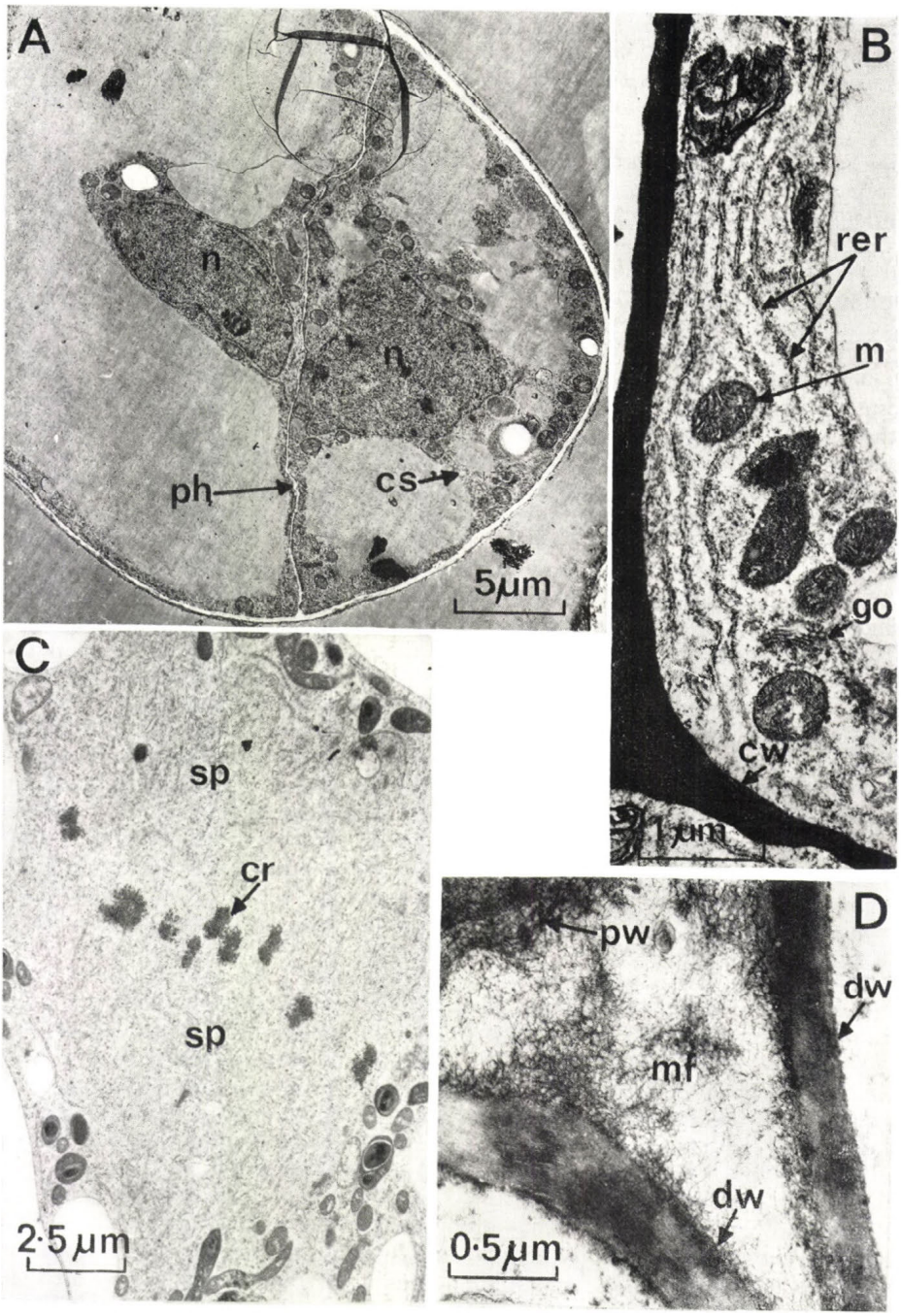
Plate 1. Structure of stationary phase cells used to initiate cultures. A, 28-day cells—note lobed nucleus, large central vacuole; B, Detail of cytoplasmic lining of early stationary phase cell (21 days); C, Plastids packed with starch grains; D, Multivesicular body; E, Microbodies

Key. cr, crystalloid of microbody; cw, cell wall; er, endoplasmic reticulum; m, mitochondria; mb, microbody; n, nucleus; p, plastid; pr, protein or lipid body; rer, rough endoplasmic reticulum (showing ribosomes); sg, starch grain; t, tonoplast; v, vacuole

cell wall (Plate 1A). The nuclei are extensively lobed (Plate 1A and B) and organelles tend to be concentrated in the cytoplasm adjacent to the nucleus; mitochondria along with other organelles often occur within the nuclear invaginations. Organelles occur at relatively low density. The mitochondria are mainly of the light matrix type. Ribosomes are associated with the sparse endoplasmic reticulum profiles, some are organized into small polysomes. Golgi bodies are scattered in the cytoplasm. Large amyloplasts are also usually present (Plate 1C). There can usually be observed protein or lipid bodies (2–4 μm diam.) and multivesicular bodies (Plate 1D and Plate 4A) corresponding to those described by Jensen (1965). One organelle which is prominent in the stationary phase cells and which declines markedly in frequency during lag phase and reappears again as stationary phase is approached is the crystalloid microbody, 0·5–1·0 μm diam. (Plate 1E). The structure of these microbodies, which contain a characteristic crystalloid and are bounded by a unit membrane, has been previously described in some detail (Davey and Street, 1971). Such bodies have been observed in many different plant cells and in cells from suspension cultures of *Eucalyptus camaldulensis* (Cronshaw, 1964). In contrast to the crystals of animal microbodies (Hruban and Recheigl, 1969), the polytubular lattice here appears to be an open structure in which the tubules do not form wall of a large central tubule. The function of these microbodies is uncertain but they seem to be characteristic of 'aged' cells and Frederick and Newcomb (1969) have suggested that the crystalloid is a catalase deposit. They may, however, contain reserve proteins.

During lag phase, preparatory to the onset of cell division, there is a massive increase in the volume of cytoplasm per cell and associated with this the development of transvacuolar strands of cytoplasm and large increases in the numbers of mitochrondria, ribosomes, ER profiles, Golgi bodies and plastids. Dumbel-shaped mitochondria have been observed suggesting mitochondrial division. Cristae are narrow and clearly defined. Proplastids as well as amyloplasts are always observed. ER membranes often show strikingly parallel arrangement (Plate 2B). Golgi bodies are surrounded by clusters of Golgi vesicles (Plate 3B). Ribosomes occur all along the ER profiles (Plate 1E and Plate 2B) and polysomes seem to involve large numbers of ribosomes.

Cell division involves segmentation of the initial cells into 2, 4 or 6 cells initially retained within the old cell wall; the daughter cells although each bounded by a complete new wall are for a time enclosed within the parent wall of the inoculum cell (Plate 2D). This parent wall is then ruptured and the aggregate of new cells released; during this process the breaking wall may become teased out to show its microfibrillar structure (Sutton-Jones and Street, 1968). Preparatory to cell division the nuclei round off and a protoplasmic bridge (a phragmasome) is developed across the cell, into which the nucleus migrates (Plate 2A). During the transient period of exponential growth, many cells are observed in mitosis (Plate 2C). The cell plate develops between the two daughter nuclei and spreads laterally to divide the cell (Plate 3A). Golgi bodies aggregate in the region of cell plate formation and the plate appears to arise by fusion of Golgi vesicles, their membranes fusing to form the plasmalemmae of the daughter cells (Plate 3A and Plate 3B). Microtubules are prominent, presumably corre-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sponding to the spindle fibres (Plate 3C). Microtubules can also often be observed adjacent to the cell walls (Plate 3D) and may be involved in the synthesis of new wall material. The newly formed wall between the daughter cells follows a characteristic zig-zag course (Plate 4A).

With the continuation of cell division, aggregates build up in which the cells are interconnected by plasmadesmata (Plate 4B). Characteristic internal wall thickenings, on the walls delimiting the surface of the aggregate in contact with the medium, are often to be observed. These thickenings are commonly ridge-like (Plate 4E) but in some cases take the form of a labyrinth of microfibrillar material connected to the original cell wall by arm-like processes. The cytoplasm adjacent to these thickenings is usually rich in Golgi bodies and in electron opaque globules (Golgi vesicles?). The significance of these wall thickenings is uncertain. They resemble wall modifications observed by Bowes (1969) in callus of *Andrographis paniculata* and the wall invaginations of 'transfer' cells (Gunning et al., 1968; Gunning and Pate, 1969). They may, therefore, serve to greatly increase the plasmalemma area over which solute transfer occurs between the aggregate and its bathing medium.

As cell division rate declines rapidly and the culture approaches its stationary phase, the cells enlarge and the aggregates break up. As this occurs the central vacuole comes to occupy an increasing proportion of the cell volume, organelles with the exception of microbodies become less abundant and the nuclei take on their highly lobed appearance. Some mitochondria show swollen cristae and an electron opaque matrix. Starch which may have been sparse during the phase of rapid cell division again accumulates in the amyloplasts (although it does not persist if subculture is delayed and the cells draw upon their endogenous carbohydrate reserve). As cells enter stationary phase structures which we have termed paramural bodies and which may have their origin in invaginations of the plasmalemma (plasmalemmasomes–Marchant and Robards, 1968) can ofter be observed associated with the internal surface of the cell walls (Plate 4C and Plate 4D). It may be that it is from these structures that the multivesicular bodies arise and these latter are also seen adjacent to the vacuole in cells entering stationary phase. The function of these paramural and multivesicular bodies remains obscure.

The plastids are oval or elongated and occasionally cup-shaped and often resemble the mitochondria in size (Plate 5A). They contain a poorly dif-

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Plate 2. Cell division and cell separation. A, Two daugther cells showing new cell wall passing along line of phragmasome; B, A region of the cytoplasm in a cell from an actively dividing culture showing profiles of rough endoplasmic reticulum parallel to the cell wall; C, Cell in metaphase of mitosis; D, Separation of daughter cells still enclosed within the old wall of the stationary phase cell

Key. cr, chromosomes; cs, cytoplasmic strands crossing vacuole; cw, cell wall; dw, wall of daugther cells; go, Golgi body; m, mitochondrion; mf, microfibrillar material teased out as cell walls separate; n, nucleus; ph, phragmasome containing new cell wall; pw, wall of 'parent' (stationary phase cell); rer, rough endoplasmic reticulum; sp, spindle

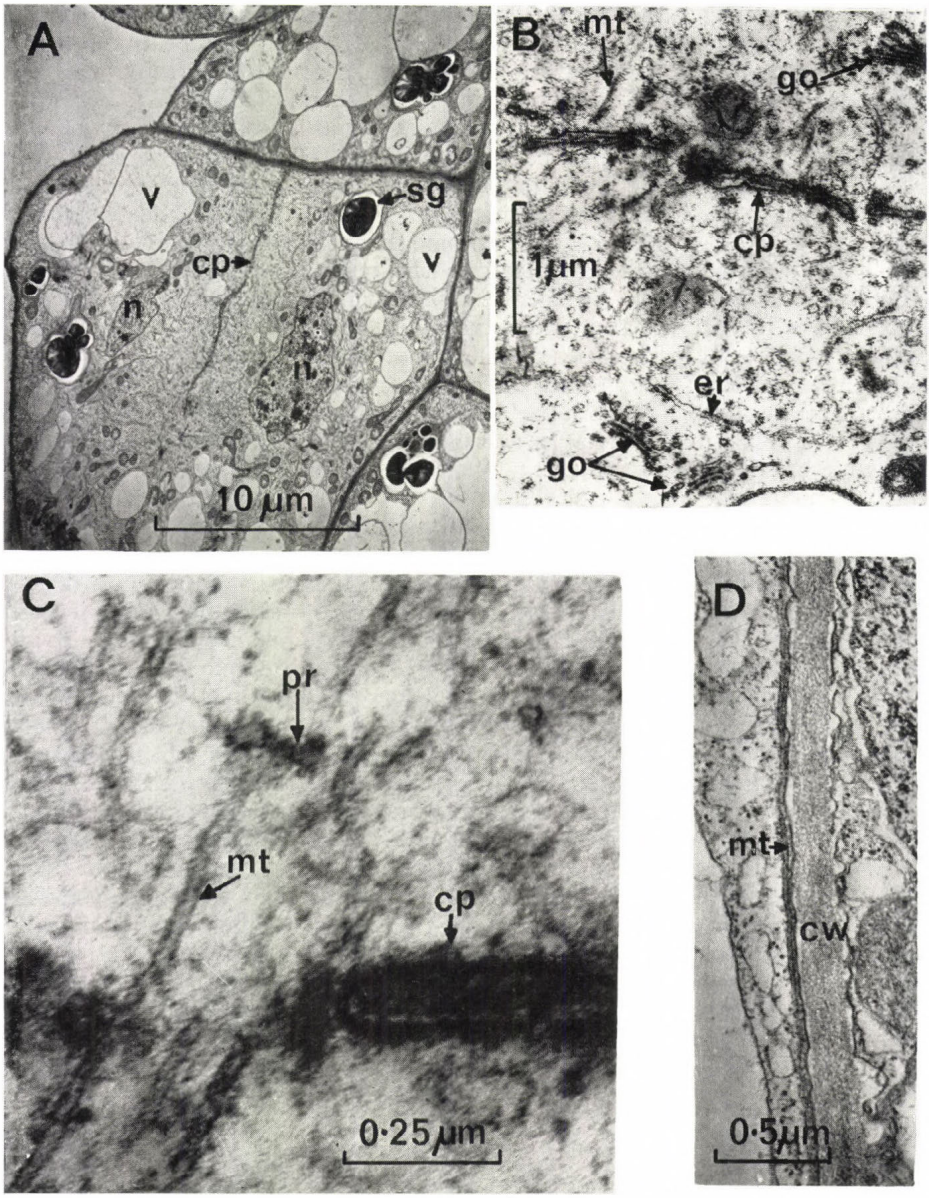


Plate 3. Cell plate formation. Microtubules. A, A cell of an aggregate in the process of cytokinesis. Cell plate developing between daughter nuclei; B, Higher power in region of cell plate formation. Many ribosomes present; C, Microtubules passing through gaps in cell plate; D, Microtubule parallel to cell wall

Key. cp, cell plate; er, endoplasmic reticulum; go, Golgi body; mt, microtubule; n, nucleus; pr, polyribosome; sg, starch grains; v, vacuole

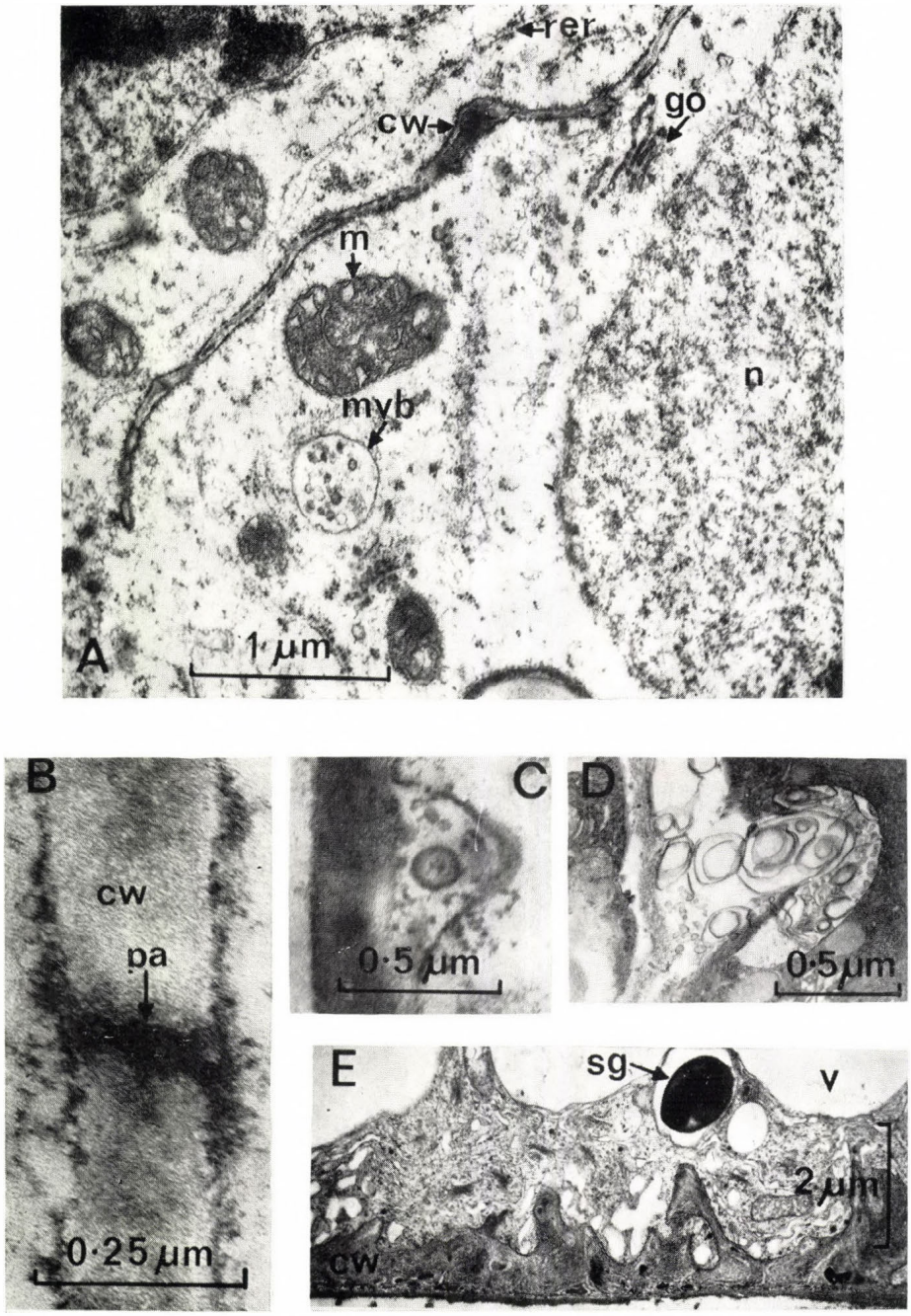
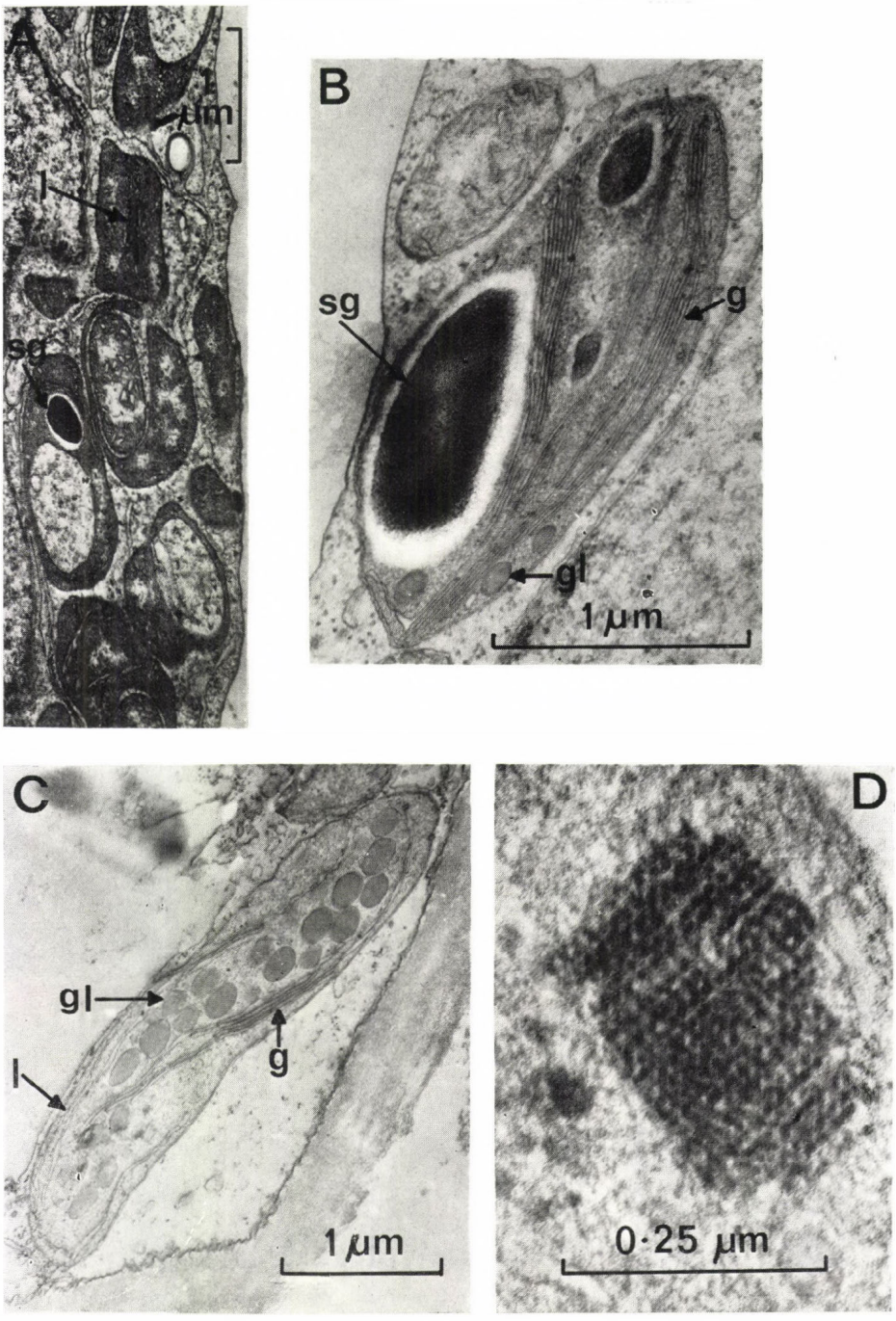


Plate 4. Cell walls and associated structures. A, Wavy newly formed cell wall; B, Plasmadesmata; C and D, Paramural bodies; E, Peg-like internal projections directed inwards on external cell walls of cell aggregates

Key. cw, cell wall; go, Golgi body; m, mitochondrion; mvb, multivesicular body; n, nucleus; pa, plasmadesmata; rer, rough endoplasmic reticulum; sg, starch grains; v, vacuole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ferentiated lamella and vesicle system and globules (which are larger and less electron dense than the osmophilic globules of chloroplasts) (Plate 5C). The plastids increase enormously in size when swollen with starch grains (Plate 1C). Occasional inclusions in the plastids strongly suggestive of phytoferritin (Hyde et al., 1963) have been observed (Plate 5D). The presence of 2,4-dichlorophenoxyacetic acid (2,4-D) at 1 mg per litre in our standard culture medium effectively suppresses chloroplast development even when the cultures are incubated in the light. If however, we replace the 2,4-D by 2·5 mg per litre α-naphthalene acetic acid (NAA) and culture at a fluourescent light intensity of ca. 11,000 lux, the suspension becomes green. In such a suspension some of the cells contain well differentiated chloroplasts (Plate 5B), but the same cells also contain undifferentiated plastids, and many cells remain hyaline.

The cells so far described were from cultures grown in a medium containing an initial concentration of sucrose of 2 per cent; this medium becomes depleted of sugar by day 18–20 of incubation under the conditions of culture described. With the exception of the internal wall projections reported to occur within the boundary walls of the cell aggregates, and less frequent projections on walls within aggregates, the cell walls of suspension-grown sycamore cells remain thin; they lack a defined secondary wall. The cells expand but do not embark upon a recognizable pathway of differentiation (leaving aside here the very important problem of chloroplast differentiation which we have discussed elsewhere) (Davey et al., 1971). The cultures do, however, lose considerable amounts of hemicellulose to their culture medium (Street et al., 1968), and by enhancing the initial levels in the culture medium of sucrose (concentrations up to 15 per cent will support growth) and of auxin (2,4-D up to 10 mg per litre) it has been shown that the cultures produce considerable amounts of lignin (Carceller et al., 1971). There is, however, no associated secondary wall formation or cell wall lignification. The lignin is partly retained within the cell protoplasts, partly deposited between cells associated in aggregates and partly released into the medium as thin plates of material. When cells active in lignin formation are examined in the electron microscope plates of electron opaque material between the cells are observed (Plate 6C) and dense globules are being secreted into the vacuoles via the tonoplast (Plate 6A and Plate 6B). These can be interpreted as corresponding respectively to the extra- and intracellular lignin. The cultured cells can apparently synthesise the matrix material ('hemicellulose') and the lignin but not the cellulose framework for a secondary wall.

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Plate 5. Various aspects of plastid morphology observed in cells on the 14th day of culture. A, Plastids with poorly developed lamella system and showing deep invaginations; B, A chloroplast in cells cultured with 2·5 mg per litre NAA instead of 1·0 mg per litre 2,4-D as auxin; C, A plastid in a colourless cell from a culture with 2·5 mg per litre NAA instead of 1·0 mg per litre 2,4-D as auxin; D, Phytoferritin aggregate in the stroma of a plastid
Key. g, granum; gl, globule in stroma; l, lamellae; sg, starch grain

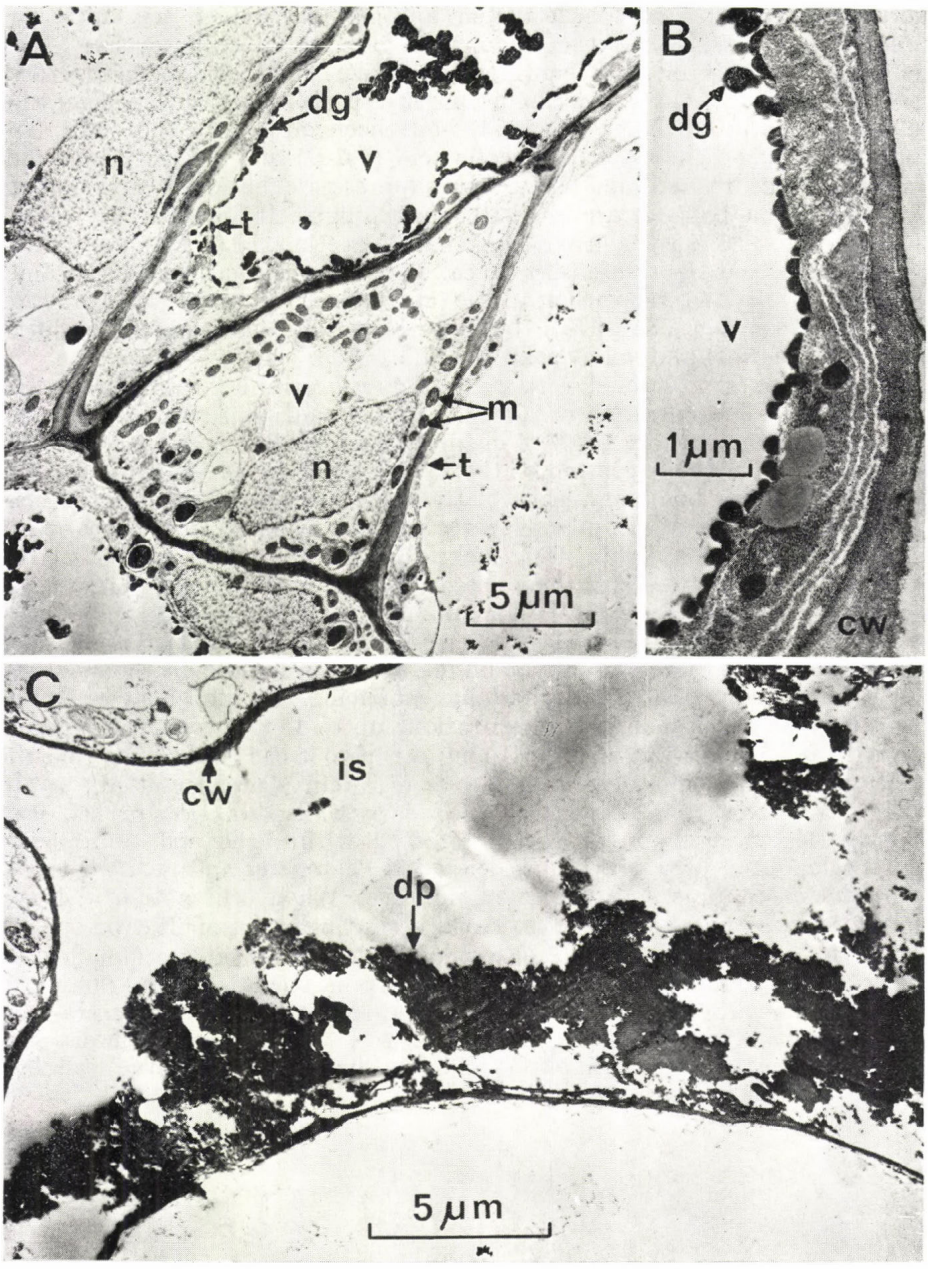

Plate 6. Cells known to be producing lignin. A, Cell showing appearance of electron dense globules in central vacuoles; B, Higher power showing clustering of electron dense globules at tonoplast, C, Presence of plates of electron dense material in intercellular spaces within a cell aggregate

Key. cw, cell wall; dg, electron dense globules; dp, electron dense plates; is, intercellular space; m, mitochondrion; n, nucleus; t, tonoplast; v, vacuole

DISCUSSION

Some aspect of the fine structure of sycamore cells growing in suspension culture have also been reported by Roberts and Northcote (1970). Their observations confirm these now reported particularly in regard to phragmosome and cell plate development, and with regard to the occurrence of microbodies and of phytoferritin in the plastids.

Other studies are mainly related to the fine structure of callus cultures growing in solid media. Such studies reveal a close similarity between suspension cultured cells and the cells responsible for the growth of undifferentiated calluses (Davey, 1970). There is clearly a close similarity between the structure of cultured plant cells and that of parenchymatous cells developing immediately behind the apical meristems of shoots and roots. However, and this applies particularly to highly dispersed suspension cultures like those of sycamore, such cultured cells do not follow the specific pathways of differentiation which lead to the emergence of the characteristic tissue cells of higher plants. We have referred to two examples, in a cell suspension, of aberrant rather than normal differentiation, lignin synthesis (Carceller et al., 1971) and greening (Davey et al., 1971). Further work is needed to expose the missing regulatory factor(s) in such cases; and in this electron microscopy is going to be essential to describe the abnormality at the structural level and to recognize any modifications which normalize the differentiation pathway .

Hitherto, we have in our studies with cell suspension been confined to cells propagated by batch culture. Cells, so cultured, are in a continuous state of change. Recently successful techniques of open continuous culture have been developed (Wilson et al., 1971) and have made possible the achievement of steady states of growth and metabolism. Such steady states can be achieved over a wide range of growth rates and nutritional conditions and the cells in these different steady states are strongly contrasted in cell size, cellular composition and physiological activity. Great interest will now attach to fine structural studies on such steady state cultures and on the sequence of structural changes which occur when the culture is in transition from one steady state to another.

*

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